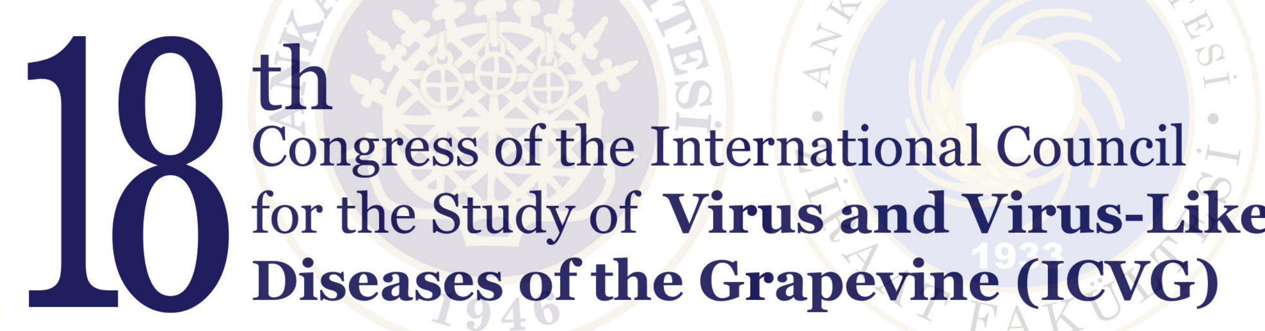
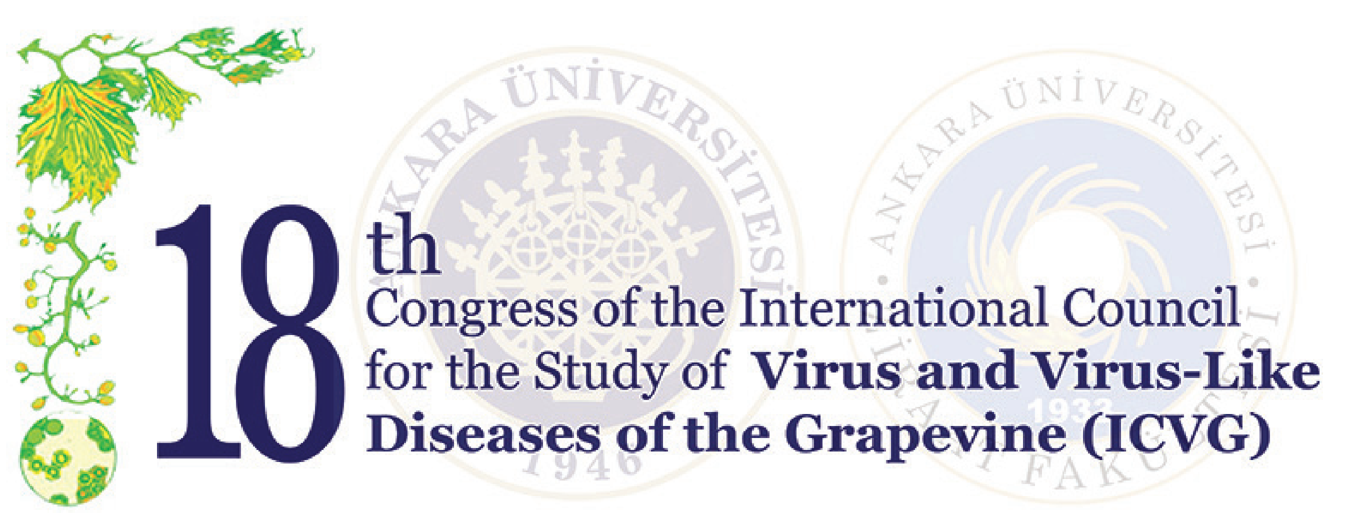


18th Congress of the International Council for the Study of Virus and Virus-Like Diseases of the Grapevine (ICVG)

PROCEEDINGS

September 7,11, 2015 Ankara - Turkey



Dear Colleagues

It will a great pleasure for me to invite you to 18th Congress of the International Council for the Study of Virus and Virus-like Diseases of the Grapevine (ICVG) which will be held in Ankara on 7-11 September, 2015. This will be the first meeting of ICVG in Turkey. Its venue will be Sheraton Hotel in Ankara. We feel honored to host this meeting, and hope that you will enjoy the scientific presentations, networking opportunities, field trips and our beautiful city of Ankara.

Hoping to seeing you in Ankara,

Prof. Dr. Filiz ERTUNÇ

Chair,18th ICVG Organizing Committee

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10. Volos (Greece) 3-7 September 1990
11. Montreux (Switzerland) 5-10 September 1993
12. Lisbon (Portugal) 28 September - 2 October 1997
13. Adelaide (South Australia) 12-17 March 2000
14. Locorotondo (Italy) 12-17 September 2003
15. Stellenbosch (South Africa) 3-7 April 2006
16. Dijon (France) 31 Aug - 4 Sep 2009
17. Davis (California) 7-14 October 2012
18. Ankara (Turkey) 7-11 September 2015

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PLENARY LECTURES

Viticultural properties and germplasm profiles of Turkey

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INTRODUCTION

Turkey is one of main gene center and domestication area of wild Eurasian grapes Vitis vinifera ssp. slyvestris together with a great biodiversity its cultivated form Vitis vinifera ssp. sativa .

Climatic variable and landforms offer very convenient conditions for grape growing in Turkey. According to the latest data of FAO (Anonymous,2014 a), the total area of vineyards is about 462.000 hectares which is the fifth greatest grape land of the world, coming after Spain, France, Italy and Republic of China. Compared to the global data, Turkey's vineyards represent 6.63% of the world's viticultural areas. Grape production of the country is about 4.2 million tones. Among the world grapevine producing countries, this value is placed in the sixth rank coming after Italy, China, USA, France and Spain (Table 1).

Table 1: Vineyard area and grape production in the top ten countries of the world

		Area (ha)		Country	Production (ton)
1	Spain	943 000	1	China	9 699 267
2	France	760 805	2	USA	6 661 820
3	Italy	696 756	3	Italy	5 819 010
4	China	602 800	4	France	5 338 512
5	Turkey	462 295	5	Spain	5 238 300
6	USA	389 349	6	Turkey	4 234.305
7	Argentina	220 000	7	Chili	3 200 000
8	Iran	215 000	8	Argentina	2 800 000
9	Chili	204 000	9	Iran	2 150 000
10	Portugal	179 500	10	Australia	1 656 621
	World Total	**6 969 373**		**World Total**	**67 067 129**

Grape production is an important horticultural sector in Turkey's agriculture. As it is seen in Table 2, vineyards occupy approximately 2% of total plant cultivation area. In horticultural plantations the ratio of vineyards is about 11.7 % (Anonymous, 2014 b).

Table 2: Plant cultivation area of Turkey (1000 ha)

Field Crops		Horticultural Crops				Total	
Cultivated	Fallow	Vegetables	Fruits	Vineyards	Olive	Area for Horticulture	Area for Plant Production
15 634	4286	827	1 856	462	814	3 959	23 879

Nearly all regions of Turkey have favorable ecological conditions for viticulture. Among the nine agricultural regions, Aegean is the leader in both area and production. Mediterranean, Mid-south and South-east Anatolia regions are the following regions. However, at the high altitudes of East Anatolia and East Black Sea region where the rainfall is over 1000 mm viticulture has minor importance.

Grape is the most produced fruit having 21% of total fresh fruit production. In the worldwide the distinctive characteristic of Turkey's grape production is based on table grapes, seedless and seeded raisins and traditional must products. Wide range of harvesting time from late May to mid November caused by regional climatic differences offers marketing advantages in table grape growing. In the world Turkey is the biggest seedless raisin grapes producer in common with USA. Recently traditional must products such as grape molasses, grape molasses with walnut, dried layers of molasses, grape juice and certain similar products come to be well known in the global market. Seeded raisin grape production is another important traditional field of viticulture. Only about 3% of total production

was processed for wine whereas historically Turkey is one of the homelands of wine making. This contradictory result can be explained by religious and social habits of the public.

The rate of income derived from grape and products is about 13% of total agricultural trade and 0.5 % of total enterprise. The main exporting item belongs to the seedless raisin grape Sultani. Every year, Turkey export almost 90% of the total raisin grape production with respect to yield and market conditions. Even though table grapes can be exported in large quantities it is difficult to say that Turkey can use this capacity efficiently (Anonymous, 2014b)

Grapevine germplasm

Numerous archeological findings and researches which are based on genetic approaches have been revealed the importance of Anatolia and surrounding geographic area on domestication and cultivation of Vitis vinifera L. (Vouillamoz et al., 2006). Table 1 has been demonstrated the summary of studies have been recognized the natural localities of wild grapevine (Vitis vinifera ssp. slyvestris) in Turkey (Uzun and Bayır, 2010).

Table 3: Distribution area of Vitis sylvestris in Turkey.

Province	Reference
Artvin, Hopa, Rize, Trabzon, İstanbul, İzmir, Muğla, Silifke-Anamur	Oraman, 1952
Köyceğiz, Fethiye, Gümüldür	Alleweldt, 1965
Kırklareli, Çanakkale, İstanbul, Sakarya, Amasya, Merzifon, Kütahya, Elazığ, İzmir, Muğla Antalya, Seyhan, Hatay	Davis, 1967
Trabzon, Bayburt, Gümüşhane, Diyarbakır, Bitlis, Adıyaman, Kahta, Maraş	Schumann,1977
Antalya	Uzun *et al.* ,1998
Mersin, Antalya, Muğla, Tekirdağ, Çanakkale	Çelik *et al.*, 2005

On the other hand, the world well known ancient grape varieties, such as Sultani, Öküzgözü, Boğazkere, Kalecik Karası have been originated in this area. By the national project was started at 1965, 1437 grape genotypes have been transferred into National Collection Vineyard in Tekirdağ. Accessions have already been analyzed at morphological and molecular level in order to catalog biodiversity (Boz et al., 2012).

Since years Black sea cost has been hosted Vitis labrusca vines which are grown on trees and home fences. Their grapes are lovingly consumed and conserved. Also biodiversity in the species is used as a source for resistance breeding studies.

In the ongoing grape breeding programs, totally 132 clones have been selected. As a result of hybridization studies 16 new grape cultivars were released.

Vineyard profile and propagation material

However in recent viticultural developments, vineyards are established on proper rootstocks, in some parts of Turkey grapes are grown on their own roots. Rootstock varieties and number are very dynamic. In vineyards either head pruning or modern training systems are used with respect to economic and regional characteristics.

Organic grape growing is the newly improving area in viticulture. Undoubtedly organic seedless raisins are the most important organic agricultural product.

Propagation materials can be produced by public or private nurseries. Propagation of healthy planting materials is under the control of Ministry of Agriculture. In recent years substantial efforts have been realized at all levels of propagation system to improve the health status of grapevine planting materials.

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Grapevine Virology: A historical account with an eye to the studies of the last 60 years or so

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As reviewed by Martelli (2014), there are some 30 or so recognised virus and virus-like diseases of grapevines, which are characterized by a variety of symptoms, such as stunting, reduced vigour, malformations of the leaves and twigs, foliar discolourations (reddening, chlorotic or bright yellow mottling, ringspots and line patterns), grooving and/or pitting of the woody cylinder. The productive life of the vineyards can be shortened and the quantity and quality of the yield badly affected. Prevailing agents of the three major disease complexes, whose history is reviewed hereafter, are viruses with isometric particles, the most relevant of which are transmitted by nematodes (nepoviruses), and viruses with filamentous particles transmitted by pseudococcid mealybugs and soft-scale insects (closteroviruses and vitiviruses) or by eriophyid mites (trichoviruses). Infected propagation materials are the major responsible for long-distance dissemination of the diseases, several of which have now a worldwide distribution and have entered areas where the grapevine industry is expanding.

This is, in summary, the current situation. However, how was it in the past, and when and where from the sanitary problems originated?

The first descriptions of an alarming degenerative condition (infectious degeneration) of grapevines date back to the second half of the 19th Century. These early records were from European countries: France (Cazalis-Allut, 1865), Austria (Rathay, 1882), Germany (Cholin, 1896), and Italy (Baccarini, 1902). In a few decades, evidence was gathered that this disease had a patchy distribution in the field, was graft-trasmissible and no infection occured when the soil was heated at 120°C (Schiff-Giorgini, 1906; Pantanelli 1910, 1917; Petri 1918). On these bases, Petri (1929) endorsed Baccarini's (1902) early suggestion of the putative viral origin of the disease in question.

Notwhitstanding the relevance of infectious degeneration, after the early 1900s upsurge of interest for it there was no much action in Europe and elsewhere up to the mid 1950s, when the studies carried out in California (Hewitt, 1954) revealed that grapevines are affected by a number of different virus and virus-like diseases, and provided a detailed description of the relative symptomatologies. This was soon followed by the demonstration that fanleaf (i.e. the same as the European infectious degeneration) is indeed a soil-borne disease transmitted by the longidorid nematode *Xiphinema index* (Hewitt *et al*., 1958) and, shortly afterwards, that the putative agent of fanleaf is a mechanically transmissible nepovirus (Cadman *et al*., 1960). These papers revived the attention for the long neglected viral problems of the viticultural industry, first in Europe, then in the rest of the world.

In May 1962, a group of American and European plant pathologists, following an encounter in France with members of the Office International de la Vigne et du Vin (OIV) in which very diverging views on virus diseases of grapevines had emerged, decided to create a study group independent from OIV, denoted "International Council for the Study of Virus and Virus-like Diseases of the Grapevine" (ICVG), where to discuss freely on their researches and relative results. Father founders of ICVG were scientists from the USA (W.B. Hewitt), France (A. Vuittenez), Germany (W. Gërtel), Italy (E. Baldacci and A. Ciccarone), Portugal (H. Dias) and Switzerland (R. Bovey) (Bovey and Gugerli, 2003).

ICVG has given a tremendous impulse to virological studies. Since 1962, some 62 different viruses have been identified in grapevines (*Vitis* and *Muscadinia*), about one thrid of which (17 viruses) are associated with the three major disease complexes known as: (i) infectious degeneration (11 European/Mediterranean nepoviruses) and decline (5 American nepoviruses); (ii) leafroll (5 viruses); (iii) rugose wood (6 viruses) (Table 1).

Infectious degeneration/decline

As mentioned, recognized and putative agents of infectious degeneration/decline are viruses with isometric particles classified in the genus *Nepovirus,* many of which (8 of those infecting vines) have a recognized nematode vector. These viruses have a bipartite single-stranded, positive-sense RNA genome, the complete sequence of 12 of them (ArMV, CLRV, GARSV, GBLV, GCMV, GDefV, GFLV, RpRSV, SLRSV, TBRV, TRSV, ToRSV) has been determined (Martelli, 2014). A comparative analysis of these sequences disclosed that recombination at the level

of RNA-2 is an efficient evolutionary mechanism of these viruses that results in the emergence of interspecific hybrids (Olivier *et al*., 2010) and novel viral species. The latter is the case of: (i) *Grapevine chrome mosaic virus*, a recombinant between *Tomato black ring virus* and *Grapevine Anatolian ringspot virus* (Digiaro *et al*., 2015); (ii) *Grapevine deformation virus*, a recombinant between *Grapevine fanleaf virus* and *Arabis mosaic virus* (Elbeaino *et al*., 2012).

Viruses involved in degenerative diseases (fanleaf and the like) are referred to as European/Mediteranean nepoviruses because, except for GFLV which has a man-fostered worldwide distribution, they occur in this geographical area and have vectors sharing the same territorial distribution (Martelli and Taylor, 1990). Thus, degenerative diseases and relative agents prevail in Continental and Mediterranean Europe where they are likely to have originated, whereas other diseases denoted “grapevine decline”, their eliciting viruses and vectors are found primarily in North America.

Based on the above, it was hypothesized that degenerative diseases occured in Europe before the arrival of phylloxera (Daktulosphaira vitifoliae Fitch.) in 1863, thus are native to the Old World. This likelihood is supported by additional evidence: (i) old records in the European literature describing the symptoms of the disease; (ii) discovery in a Sicilian herbarium of the second half of the 19th century of dried grapevine leaves with symptoms identical to those currently visible in vines infected by chromogenic and distorting strains of GFLV (Martelli and Piro, 1975); (iii) old paintings, [e.g. Pompeii frescos (79 AD) and a painting by Caravaggio (1600)] depicting distorted grapevine leaves resembling those from fanleaf-diseased plants; (iv) GFLV, the major causal agent of degeneration, is serologically related to ArMV, an European nepovirus with which it can recombine to give rise either to new “pathotypes” [e.g. chromogenic virus strains (Elbeaino et al., 2014)] or to novel grapevine-infecting viral species [e.g. Grapevine deformation virus (Elbeaino et al., 2012)]; (vi) Xiphinema index, the vector of fanleaf, is a nematode thought to be native of Asia minor (ancient Persia) (Hewitt, 1968; Mojtahedi et al., 1980), whose eastern origin was confirmed through the analysis of mitochondrial genes and miscrosatellite loci (Villate, 2008); (vii) GFLV occurs in phylloxera-free countries (e.g. Cyprus, Armenia, parts of southern Turkey, some Aegean Greek islands) (Martelli, 2104), where American rootstocks have not been introduced.

Evidence of the American origin of the decline syndromes rests on their almost exclusive occurrence in *Vitis vinifera* and *V. labrusca* grown in the northern United States and Canada, in the origin of the eliciting viruses (ToRSV, TRSV, PRMV) whose presence in other geographical areas is due to accidental introductions, and in the distribution of their vectors, which are largely restricted to North America (Martelli and Taylor, 1990; Martelli and Uyemoto, 2011).

Leafroll

Graft-transmission of leafroll from grape to grape was first obtained in Germany by Scheu (1936). A decade later Harmon and Snyder (1946) described in California a graft-transmissible disease of cv. Emperor called “White Emperor” which, after an additional decade, and again in California, was shown to be the same as “Leafroll” (Goheen *et al*.,1958). Thus, in the early 1960, the infectious nature of leafroll was established, but its aetiological agent was still unknown. The discovery of filamentous virus-like particles in the sieve tubes of German vines affected by yellows (Mengden, 1971) was largely unnoticed, notwithstanding the fact that the similarity with citrus plants infected by the closterovirus *Citrus tristeza virus* was striking. The breakthrough in leafroll aetiology came a few years later when closterovirus-like particles were recovered in Japan from vines with leafroll symptoms and their presence was linked with the disease (Namba *et al.,* 1979).

The first partial characterization of two serologically different leafroll-associated closteroviruses came from Switzerland in 1984. These viruses were referred to as “type I” and “type II” (Gugerli et al. 1984). This was the beginning of the nomenclature based on the use of numbers. In the years that followed the number of putatively new closterovirus species found in vines with leafroll symptoms increased in a disorderly way, so as to call for a revision of their nomenclature. The name of virus species was thus determined to be “Grapevine leafroll-associated virus” followed by an Arabic numeral, e.g. GLRaV-1, -2 and so on (Boscia et al., 1995).

For many years leafroll was thought not to be spreading in the field (e.g. Goheen, 1989), and the reports from different countries (e.g. Dimitrijevic, 1973) that this was not the case were not paid much attention. A leap forward was made when, based on the evidence that *Grapevine virus A* (GVA) which at that time was classified as a “short closterovirus”, was transmitted by the mealybug *Pseudococcus longispinus* (Rosciglione *et al*., 1983). Investigations carried out in Switzeland showed that GLRaV-3 is vectored by *Planococcus ficus* (Rosciglione and Gugerli, 1989). Transmission is non specific (multiple vectors) and semi-persistent [transmission parameters determined for GLRaV-3 are: acquisition access period = 1 h; inoculation access period = 30 min) (Krüger *et al*., 2006; Almeida *et al*., 2013)]. Recognized vectors are: *Heliococcus bohemicus, Phenacoccus aceris, Ps. affinis, Ps.calceolariae, Ps. viburni, Ps. maritimus, Ps. comstocki, Ph.aceris, Pulvinaria vitis, Neopulvinaria innumerabilis, Parthenolecanium corni* (GLRaV-1); *Planococcus ficus, Pl. citri*, *Pseudococcus longispinus. Ps. calceolariae, Ps. maritimus, Ps. affinis Ps. viburni, Ps. comstocki, Phenacoccus aceris, Parthenolecanium corni, Neopulvinaria innumerabilis, Pulvinaria vitis, Coccus hesperidium, C. longulus, Saissetia, Parasaissetia, Ceroplastes*

(GLRaV-3); *Ps. longispinus, Pl. ficus, Ph. aceris* (GLRaV-4 and several of its strains).

Closteroviruses have very flexuous filamentous particles with distinct cross-banding, are members of the family *Closteroviridae* and are classified in four genera: *Closterovirus* (vectored by aphids), *Ampelovirus* (vectored by mealybugs), *Crinivirus* (vectored by whiteflies), *Velarivirus* (vector unknown). Grapevine-infecting closteroviruses belong in the genera *Closterovirus, Ampelovirus* and *Velarivirus,* and are endowed with genomes differing in size (from 13,700 to 18,500 nucleotides) and structure (from 6 to 12 genes) (Martelli *et al*., 2012). These differences derive from a modular evolution encompassing a series of successive modifications that involve the viral genome, such as gene duplication, loss of sequences due to deletion, genome bipartition, acquisition of sequences from foreign sources (e.g. other viruses, host plant, other organisms).

As hypothesized by Dolja *et al.* (2006), members of the family *Closteroviridae* have evolved from a plant-infecting filamentous alphavirus-like progenitor whose "simple" genome encoding in the order: (i) the replication-associated proteins; (ii) a 6 kDa product that is a conventional non structural movement protein; (iii) the coat protein, has undergone a progressive increase in size and complexity upon the acquisition of additional functional sequences, which resulted in the rise of the ancestors of the different genera.

Although leafroll is now one of the most widespread virus disorder of the grapevine in the world, its origin seems to hail from the Old World where the disease is likely to have occurred long before the arrival of phylloxera. Supporting evidence is: (i) old records in the Italian and French literature describing an abnormal condition of grapevines called "rossore" and "rougeau" (reddening), respectively; (ii) presence in a Sicilian herbarium of the second half of the 19th century of dried grapevine leaves reported as being affected by "rossore". These specimens show unmistakable signs of a leafroll condition, i.e. downward rolled, very heavy, thick, fractured and blackish blades (Martelli and Piro, 1975); (iii) occurrence of some of the leafroll-associated viruses (especially GLRaV-1 and GLRaV-3) in countries like Cyprus, Armenia, Yemen, China (Sinkiang), parts of southern Turkey, some Aegean Greek islands which are still phylloxera-free, thus the vines grow on their own roots (Martelli *et al*., 1994; Pio Ribeiro *et al*., 2004); (iv) Leafroll-infected vines were present among the original grape stocks imported in 1890 from Europe by the University of California (Luhn and Goheen, 1970).

There is, however, a puzzling case which is not in line with the above reconstruction. It so happens that GLRaV-2 infections have recently been recorded in American native species: (i) *Vitis californica* and natural hybrids with *Vitis vinifera* in California (Klaassen *et al*., 1911); (ii) *Muscadinia rotundidifolia* and summer grape (*Vitis aestivalis*) in Mississippi (Abou Ganem-Sabanadzovic and Sabanadzovic, 2015). One of the Mississippi virus isolates is the same as the Californian graft incompatibility inducer GLRaV-2RG (Alkowni *et al.,* 2011) which is not known to occur in Europe, whereas the other is an ordinary leafroll-inducing strain (Meng *et al*., 2005). It ensues that the presence of GLRaV-2 in summer grapes growing in a natural ecosystem (Great Smoky Mountains Natural Park) and in muscadines in a small viticultural area (Mississippi) and in the riparian vegetation of the Napa Valley (California) seems difficult to reconcile with an eastern origin of this virus. Unless in the US there is a vector (an aphid, as with other members of the genus *Closterovirus* in which GLRV-2 belongs?) able to acquire the virus from infected *V. vinifera* and tranfer it to native *Vitis* species. Should this not be the case, the notion gains strength that GLRaV-2 is a virus native to North America.

Rugose wood

Rugose wood, a graft-transmissible disease first described from Italy (Graniti and Martelli, 1965) and soon afterwards from Hungary (Martelli *et al*., 1967), is a complex disorder within which, based on the differential reactions of the indicators *V. rupestris*, LN33 and Kober 5BB (Savino *et al*., 1987), four different syndromes have historically been identified: Rupestris stem pitting (RSP), Kober stem grooving (KSG), Corky bark (CB) and LN-33 stem grooving (LNSG).

The aetiology of rugose wood remained uncertain for many years, until the recovery by mechanical inoculation from a symptomatic vine of a virus with particles resembling those of closteroviruses (Conti *et al*., 1980) provided support to its supposed viral nature. The name of this virus, which was originally denoted Grapevine stem pitting-associated virus, was later changed into *Grapevine virus A* (GVA) (Milne *et al*., 1984). Other similar viruses were soon identified in infected vines, five of which (GVA, GVB, GVD and, later, GVE and GVF) have found a taxonomic allocation in the genus *Vitivirus* (Martelli *et al.,* 1997). An additional virus, called *Grapevine rupestris stem pitting-associated virus* (GRAPsV) (Meng *et al.,* 1998), was classified in the genus *Foveavirus* (Martelli and Jelkmann, 1998).

The extant relationship between the rugose wood syndromes and their putative agents can be summarized as follows: (i) GRSPaV/Rupestris stem pitting (Meng *et al*., 1999); (ii) GVA/Kober stem grooving (Garau *et al.,*1997); (iii) GVB and GVD/Corky bark (Bonavia *et al*., 1996); (iv) no specific virus/LN33SG identified so far. Two additional vitiviruses found in vines that showed either stem pitting (GVE) or a graft incompatibility condition (GVF) have not been assigned to a specific syndrome (Martelli, 2014).

An breakthrough in rugose wood epidemiology came when GVA was experimentally transmitted by the mealybug *Pseudococcus longispinus* (Rosciglione *et al*., 1983). This represented the first evidence that pseudococcid mealybugs, till then known as DNA virus vectors, were able to transmit also RNA viruses. It was later ascertained that, as with closteroviruses, vitivirus transmission is non specific and semi-persistent (La Notte *et al.,* 1997).

Recognized vectors are the same as those reported for ampeloviruses, with which vitiviruses are aften transmitted together: *Planococcus citri, Pl. ficus, Pseudocoscus longispinus, Ps affiinis, Heliococcus bohemicus, Phenacoccus aceris, Neopulvinaria innumerabilis* (GVA); *Ps. longispsinus, Ps. affinis, Pl. ficus, Ph. aceris* (GVB); *Pseudococcus comstocki* (GVE). The vector of GVD is still unknown, the same as the vector of GRSPaV (Martelli, 2014).

Vitiviruses and foveaviruses possess very flexuous filamentous particles with a morphology recalling that of closteroviruses with which they may share a comparable evolutionary scenario. In fact, as hypothesized by Martelli *et al.* (2007), viti- and foveaviruses have evolved from a filamentous carlavirus-like ancestor with a genome made up by five genes encoding in the order, the replication-associated proteins, the movement proteins (triple gene block) and the coat protein. The acquisition in the replicase gene of the AlkB domain (a protein that removes alkylation damage) and/or the OTU domain (a protease distantly related to papain) and the 30K-like movement protein in replacement of the triple gene block, resulted in the emergence of the genera *Foveavirus*, *Vitivirus* and *Trichovirus.* Trichoviruses are not involved in any of the rugose wood syndromes but two different species, *Grapevine berry inner necrosis virus* (GBNV) and *Grapevine Pinot gris virus* (GPGV) are pathogenic to grapevines (Yoshikawa *et al*., 1997; Giampetruzzi *et al*., 2012).

Also rugose wood appears to be an “Old world” disease based on the following evidence: (i) wood symptoms described

in the French literature of the early 20th century; (ii) occurrence of the disease and some of the rugose wood-associated viruses in phylloxera-free countries like Cyprus, Armenia, Yemen, parts of southern Turkey, some Aegean Greek islands where American rootstocks have not yet been introduced (Martelli *et al*., 1994).

Admittedly, this historical evidence is less substantiated than that standing for infectious degeneration and leafroll and it may apply only in part to GRSPaV, the type species of the genus *Foveavirus* and the most widespread of the rugose wood viruses.

In fact, GRSPaV is: (i) non mechanically transmissible; (ii) may not be seed-transmitted notwithstanding its presence in pollen grains, and has no known vector; (iii) may have evolved from an ancient recombination event between a carlavirus and a potexvirus in which ORF 4 and 5 but not the 3’ non coding region of the carlavirus were replaced by those of the potexvirus (Meng and Gonsalves, 2003); (iv) the ancestor of the present day GRSPaV may have gained entrance in different *Vitis* species in the past and, while adapting to them, its genome has diverged, producing several groups of variants. Two of the four major groups of variants may be specific to *V. riparia* and *V. rupestris* (American species) whereas two other groups may be linked with *V. sylvestris* and, perhaps, *V. vinifera* (Old World species) (Meng and Gonsalves, 2007).

PERSPECTIVES

For a look to the future one could refer to a recent review by Maliogka *et al.* (2015), whose “Concluding remarks” summarize the challenges which grapevine virologists are still confronted with. The final statement of this heading sets a priority that has emerged in many grapevine-growing countries where efforts are being spent for the improvement of the industry: “*In summary, the challenge and target of future research is not so much the development of more refined and highly perfoming techniques for the recognition and elimination of viruses but, rather, the design of dependable strategies for preventing a quick sanitary deterioration of vineyards planted with costly certified materials*.

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Table 1. Grapevine-infecting viruses, their taxonomic allocation and number of species

FAMILY	GENUS	NUMBER OF SPECIES
Viruses with isometric particles and a +ssRNA genome		
SECOVIRIDAE	*Fabavirus* *Nepovirus*	1 16
BROMOVIRIDAE	*Alphamovirus* *Cucumovirus* *Ilarvirus*	1 1 2
TOMBUSVIRIDAE	*Carmovirus* *Necrovirus* *Tombusvirus*	1 1 2
TYMOVIRIDAE	*Marafivirus* *Maculavirus*	4 2
AMALGAVIRIDAE	*Amalgavirus*	1
Viruses unassigned to families	*Idaeovirus* *Sobemevirus*	1 1
Viruses with isometric particles dsRNA genome		
REOVIRIDAE	*Oryzavirus*	1
ENDORNAVIRIDAE	*Endornavirus*	2
PARTITIVIRIDAE	*Alphacryptovirus*	2
Viruses with filamentous particles and a +ssRNA genome		
CLOSTEROVIRIDAE	*Clostervirus* *Ampelovirus* *Velarivirus*	1 3 (5 strains of GLRaV-4) 1
ALPHAFLEXIVIRIDAE	*Potexvirus*	1
BETAFLEXIVIRIDAE	*Foveavirus* *Trichovirus* *Vitivirus*	1 2 5
POTYVIRIDAE	*Potyvirus*	1
Viruses with a DNA genome		
CAULIMOVIRIDAE	*Badnavirus*	2
GEMINIVIRIDAE	Undtermined	1
Taxonomically unassigned viruses		4

ORAL PRESENTATIONS

OP 01 - *Tobacco ringspot virus* in a wine grape (*Vitis vinifera*) cultivar in Washington State

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INTRODUCTION

Among the nepoviruses known to cause fanleaf degeneration or decline symptoms in grapevines (*Vitis* spp.) worldwide (Martelli 2014), *Grapevine fanleaf virus* (GFLV), *Arabis mosaic virus* (ArMV), *Tomato ringspot virus* (ToRSV), *Tobacco ringspot virus* (TRSV), *Peach rosette mosaic virus* (PRMV) and *Blueberry leaf mottle virus* have been reported in grapevines in the United States (Oliver and Fuchs, 2011). Among them, GFLV, the causal agent of grapevine fanleaf disease, was reported in wine grape (*V. vinifera*) cultivars in Washington State vineyards (Mekuria et al., 2009). During 2013 and 2014 seasons, a vineyard block planted with a red-fruited wine grape cultivar was observed with 'fanleaf-like' symptoms consisting of leaf deformation and general decline of grapevines. Since these symptoms are characteristic of diseases caused by nepoviruses, we have conducted serological and molecular diagnostic assays to identify nepovirus(es) present in symptomatic leaves. The results described below revealed the presence of only TRSV (genus *Nepovirus*, family *Secoviridae*) in symptomatic grapevines.

MATERIALS AND METHODS

Leaf samples showing reduced size, severe malformations, vein banding and chlorotic specs and rings with mild mottling were collected from six individual grapevines. These samples were tested by Enzyme-linked immunosorbent assay (ELISA) using antibodies (Agdia, Inc., Elkhart, IN, USA) specific to GFLV, ArMV, TRSV, ToRSV, PRMV, *Tomato black ring virus* and *Strawberry latent ringspot virus*. All symptomatic samples gave positive results only with antibodies to TRSV. Subsequently, total nucleic acids were extracted (Bagewadi et al. 2015) from leaves of symptomatic and apparently healthy-looking grapevines and subjected to reverse transcription (RT)-PCR using primers specific to the coat protein (CP) gene of TRSV (Digiaro et al. 2007). Amplicons were cloned into pCR2.1 (Invitrogen Corp., Carlsbad, CA) and the nucleotide sequence of two independent clones per amplicon determined. The derived nucleotide sequences were compared with corresponding sequences available in GenBank to confirm the presence of TRSV. Multiple sequence alignments and nucleotide and amino acid sequence identity levels were calculated by ClustalW and the phylogenetic analysis was performed using the neighbor-joining method in the MEGA6 analysis package (Tamura et al., 2013).

RESULTS AND DISCUSSION

Symptomatic grapevines were present in patches in the vineyard. Samples from symptomatic vines tested positive in ELISA only for TRSV. Symptomatic vines produced smaller clusters with significantly reduced size of berries compared to clusters and berries from non-symptomatic vines. An analysis of fruit from symptomatic vines indicated significant negative impacts on quality characteristics compared to fruit from non-symptomatic vines. The approximately 254 base pair DNA fragment specific to the CP was amplified in RT-PCR only from symptomatic samples. In pairwise comparisons, partial CP sequences of four independent clones obtained from two symptomatic vines showed 93.7% to 100% identity at the nucleotide level and 100% identity at the amino acid level. These sequences showed 86.2% to 96.9% identity at the nucleotide level and 95.2% to 100% identity at the amino acid level with the corresponding sequences of TRSV isolates from soybean in S. Korea (KJ556850) and USA (AY363727) and cherry in UK (Zadeh and Foster, 2003; AF461163). Phylogenetic analysis showed segregation of TRSV sequences from Washington State into two clades, indicating genetic variability among TRSV isolates (Fig.1).

TRSV has a broad host range, including several annual and perennial crops of economic importance (Wilcox et al., 2015).TRSV was previously reported in grapevines in New York State (Gilmer et al. 1970; Uyemoto et al., 1977) and in Oregon State (R.R. Martin, personal communication). To our knowledge this is the first report of TRSV in Washington State vineyards. Further studies on distribution and spread of TRSV, relative to GFLV reported earlier (Mekuria et al., 2009), are in progress for implementing strategies to manage nepoviruses in Washington State vineyards.

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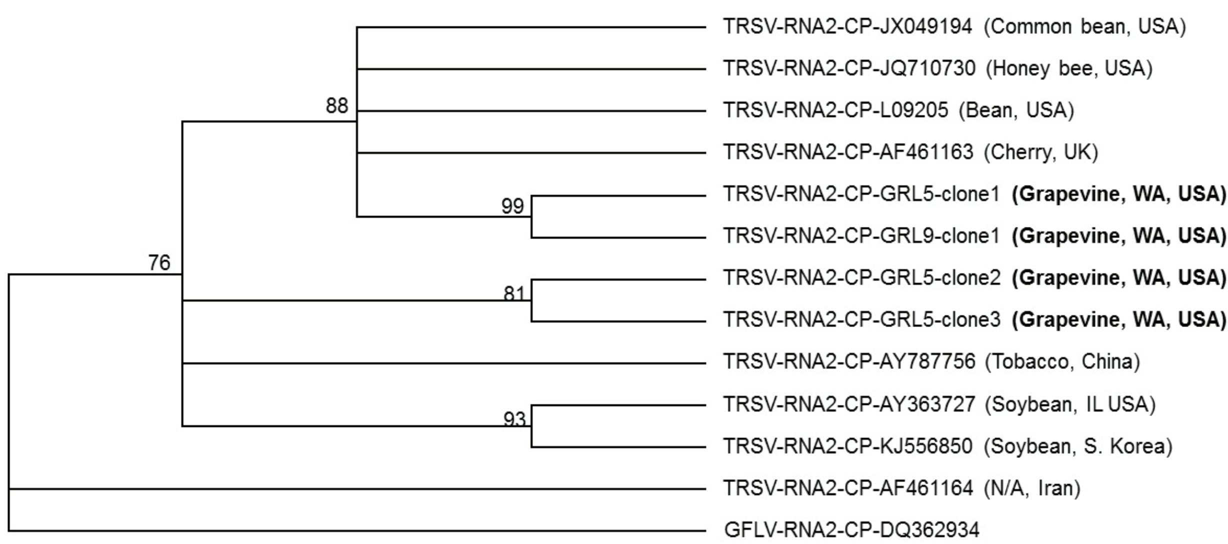

Figure 1. Phylogenetic analysis of *Tobacco ringspot virus* (TRSV) isolates based on partial coat protein (CP) sequence. The consensus tree topology was inferred using the Neighbor-Joining algorithm implemented by MEGA6. The tree was rooted by using *Grapevine fanleaf virus* (GFLV) CP sequence as outgroup. Percent bootstrap values (1000 replicates) are given at the branch nodes. Branches corresponding to partitions reproduced in less than 70% of bootstrap replicates are collapsed. Isolates from grapevines in Washington State are in bold.

OP 02 - Determination of the distribution and genetic variation of Grapevine virus A and Grapevine fanleaf virus in vineyards of East and West Azarbaijan provinces

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INTRODUCTION

Grapevines (Vitis spp.) are affected by many viral diseases; the most harmful and widespread ones are fanleaf degeneration, leafroll, rugose wood, and fleck (Gambino and Gribaudo, 2006). Grapevine virus A (GVA) is the type species of the genus Vitivirus in the family Flexiviridae (Martelli et al., 1997; Adams et al., 2004). It is also associated with leafroll syndrome as well as with rugose wood complex Gambino and Gribaudo, 2006). Grapevine fanleaf virus (GLFV) belongs to the genus Nepovirus and the family Secoviridae (Sanfacon et al., 2009). The virus is transmitted by the dagger nematode Xiphinema index (Martelli et al., 2003).The fanleaf disease is economically one of the important diseases of the vine. The study of variability is one of the most important aspects of plant virology because strains vary in the severity of the disease they cause in the field, and this variation may be highly relevant to the development of control strategies of viral diseases. Iran is one of the most important countries in the world grape production, In addition to the cultivation of large scales, diverse varieties in Iran is also interesting. Virus and virus-like diseases causing severe damage to grapevine production every year in Iran. This study aimed to investigate on the prevalence, distribution and genetic variation of these two viruses in vineyards of East and West Azarbaijan provinces.

MATERIALS AND METHODS

During 2014, leaf petioles and cane samples from 176 symptomatic plants were collected and tested for GVA and GFLV by DAS-ELISA as described by Clark and Adams (1977), using the polyclonal antisera (IVV, Italy). Quantitative measurements of the p-nitrophenol substrate conversion resulting in yellow color were made by determining the absorbance at 405 nm (A405) in a Biotek® model spectrophotometer (Lab systems Co., Finland). The mean absorbance readings of non-infected controls were determined and twice the values were used as the positive thresholds.

RESULTS AND DISCUSSION

During summer of 2014, about 176 grapevine samples with virus-like symptoms such as leaf rolling and reddening, mosaic, fanleaf and vein banding (Fig.1) were collected from vineyards in different areas and villages of East and West Azarbaijan provinces and subjected to ELISA revealed the presence of GVA and GFLV positive samples as shown on Table 1. The results of the ELISA showed that about 46.7 and 25.33% of samples were infected with GFLV and GVA in West Azarbaijan province, respectively. This ratio for East Azerbaijan was 22.8 and 21.8% for GFLV and GVA, respectively. Our results showed high rate of virus infection in these two provinces. GFLV was more prevalent in vineyards of both provinces. Molecular characteristics of isolates of these viruses in both East and West Azarbaijan provinces is underway to determine the genetic variation. Two samples in East Azarbaijan province had mixed infection. Among the Iranian grapevine varieties were tested the lowest infection rate was recorded in angor siah variety while keshmeshi variety in both provinces had the highest infection rate.

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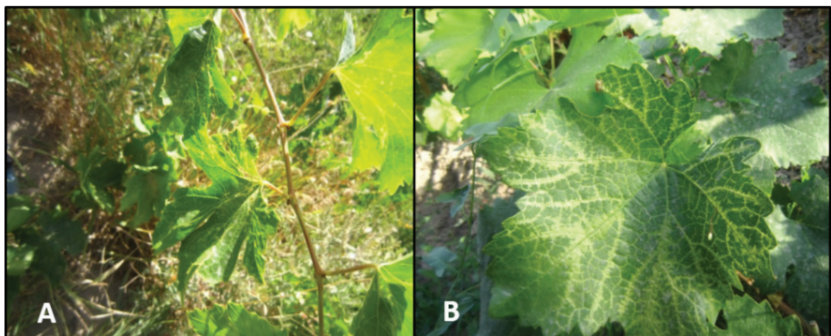

Table 1: Comparison of infection rate of two viruses in two provinces

cultivar	East Azarbaijan	West Azarbaijan	GVA		GFLV	
			East	West	East	**West**
keshmeshi	79	72	19	17	22	**35**
fakhri	6	1	2	0	1	**0**
angor siah	16	-	1	-	0	**-**
garmian	-	2	-	2	-	**0**
total	101	75	22	19	23	**35**
Infection %			21.8	25.33	22.8	**46.7**

Figure 1. Viral symptoms on grapevine leaves. A. fanleaf, B. vein banding

OP 03 - Hypersensitive-like response to *Grapevine fanleaf virus* in *Nicotiana occidentalis.*

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INTRODUCTION

Grapevine fanleaf virus (GFLV) from the genus *Nepovirus*, family *Secoviridae* is the major agent of fanleaf degeneration, one of the most damaging viral diseases of grapevine worldwide (Andret-Link *et al.*, 2004). It is specifically transmitted from grapevine to grapevine by the ectoparasitic nematode *Xiphinema index*. GFLV induces systemic infection in grapevine as well as in some *Solanaceae* species. The genome of GFLV is composed of two (+)ssRNAs, RNA1 and RNA2. Symptoms differing in type and severity consist of distorted leaves, yellow mosaic patterns, short internodes, and stunted plants, among others. Little is known about the mechanisms of GFLV symptom expression. Recently the viral determinant of mosaic on systemic-infected leaves of *Nicotiana benthamiana* and *N. clevelandii* plants was identified in the 3' coding region of the RNA-dependent RNA polymerase encoded by RNA1 (Vigne *et al.*, 2013).

In contrast to this compatible interaction on *N. benthamiana* and *N. clevelandii*, a necrotic phenotype with some characteristics of an incompatible hypersensitive reaction (HR)-like response was observed on *N. occidentalis* inoculated with GFLV-F13 but not with GFLV-GHu strains. These characteristics include necrotic spots and a partial restriction of the virus to inoculated leaves. To get insights into GFLV sequences acting as effectors involved in virulence on *N. occidentalis*, we used a reverse genetics approach with infectious cDNA clones of strains F13 and GHu, and chimeric clones derived thereof, in combination with a biochemical characterization of major hallmarks of HR interactions.

MATERIALS AND METHODS

Biological material, plant inoculation, and characterization of viral progeny

All infectious clones and recombinant procedures were performed as previously described (Vigne *et al.*, 2013). Full-length cDNA clones of GFLV-F13 and -GHu RNA1 and RNA2 were used for *in vitro* synthesis of transcripts. Four leaves-stage *Chenopodium quinoa* plants were mechanically inoculated with purified transcripts. Crude sap of infected *C. quinoa* was then used for passages in *N. occidentalis* and symptoms were monitored. Viral accumulation on inoculated and apical leaves was measured by semi-quantitative DAS-ELISA. The progeny viral RNAs were checked by RT-PCR and direct sequencing.

Characterization of the HR-like response in *N. occidentalis*

The accumulation of phytoalexins was evaluated by observation of a typical blue fluorescence surrounding necrotic spots under ultraviolet light (UV) at 3 days post-inoculation (DPI) (Chong *et al.*, 2002). Hydrogen peroxide (one of several Reactive Oxygen Species-ROS) was detected in leaves of *N. occidentalis* by 3,3'-diaminobenzidine (DAB) staining (Daudi *et al.*, 2012). Pathogenesis-related (PR) proteins were detected by western blot analyses using specific antibodies against PR1 and total proteins (Heitz *et al.*, 1994). Expression of the hsr203j gene (Pontier *et al.*, 1994) was analyzed by semi quantitative RT-PCR on total RNA extracts.

cDNAs of GFLV sequences of interest were introduced in a binary vector for agroinfiltration assays in *N. occidentalis* in order to assess plant responses upon their ectopic expression.

RESULTS AND DISCUSSION

Typical blue fluorescence rings surrounding necrotic spots were observed under UV light on leaves inoculated with GFLV-F13, suggesting the accumulation of phytoalexins (Fig 1A). In contrast, no fluorescence was observed on leaves of *N. occidentalis* inoculated with GFLV-GHu. *In vitro* transcripts of homologous combinations of RNA1 and RNA2 reproduced the symptoms observed with the wild-type parental viruses. The use of assorted transcripts showed that the formation of necrotic lesions on *N. occidentalis* mapped to RNA2 (Fig 1B). By generating recombinant RNA2, for which individual genes were swapped between F13 and GHu cDNAs, we could further map the viral determinant to one of the three coding sequence.

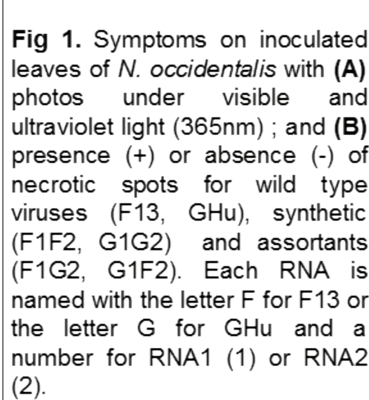
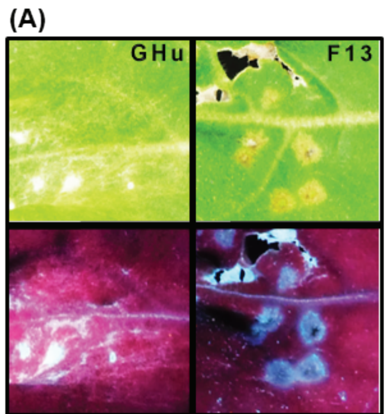


Fig 1. Symptoms on inoculated leaves of *N. occidentalis* with **(A)** photos under visible and ultraviolet light (365nm) ; and **(B)** presence (+) or absence (-) of necrotic spots for wild type viruses (F13, GHu), synthetic (F1F2, G1G2) and assortants (F1G2, G1F2). Each RNA is named with the letter F for F13 or the letter G for GHu and a number for RNA1 (1) or RNA2 (2).

(A)



(B)

Virus	Necrotic spots
F13	+
F1F2	+
GHu	-
G1G2	-
F1G2	-
G1F2	+

GFLV accumulation was low in inoculated leaves of plants exhibiting necrotic symptoms (1 mg/g) at DPI12. In contrast, GFLV accumulation was high in inoculated leaves of plants without symptoms (40 mg/g). These results suggest that the necrosis most probably restricts the virus spread and corresponds to an HR-like response of the plant. These necrotic spots also correlated with the over-accumulation of hydrogen peroxide, PR1 proteins and hsr203j transcripts.

A similar HR-like reaction was recapitulated when the F13 coding region was transiently expressed by agroinfiltration, a property that further identifies this sequence as a putative avirulence factor. The application of this transient bioassay enabled a more precise mapping to a 150 nucleotides stretch.

A comparable HR reaction was described for *Tomato ringspot virus*, another nepovirus, but the viral effector of this response remained elusive (Jovel *et al.*, 2011). Our findings are the first evidences for identifying a nepoviral coding sequence inducing an HR-like reaction in a plant host.

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OP 04 - New insights in *Nepovirus* capsid determinants involved in the transmission by *Xiphinema spp* nematodes.

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INTRODUCTION

Grapevine fanleaf virus (GFLV) and *Arabis mosaic virus* (ArMV) are the major causal agent of grapevine degeneration disease that occurs in vineyards worldwide (Andret-Link et al., 2004a). Both viruses are transmitted from grapevine to grapevine by ectoparasitic nematodes of the *Xiphinema* genus. Remarkably, *Xiphinema index* exclusively transmits GFLV while *X. diversicaudatum* vectors specifically ArMV, suggesting that highly selective molecular recognition mechanisms between virus and nematode components are involved. Structurally, GFLV and ArMV are icosahedral viruses of ca 30 nm in diameter with a *pseudo T = 3* symmetry, composed of 60 identical coat protein (CP) subunits.

The objective of our study was to identify structural domain(s) and residues within the CP that are responsible for the specificity of transmission of GFLV and ArMV by *X. index* and *X. diversicaudatum*, respectively. The identification of these viral determinants is critical to characterize the nematode determinants involved in the specific retention of GFLV and ArMV particles within the alimentary tract of *Xiphinema* spp. The final goal is to develop innovative approaches to break off nepovirus transmission.

MATERIALS AND METHODS

- Virus, host plants and virus purification: GFLV-F13 and ArMV-S were isolated from naturally infected grapevines (Andret-Link et al., 2004a). GFLV-TD is a GFLV-F13 variant in which a single Gly297Asp mutation had naturally occurred after serial mechanical inoculations on *Chenopoduim quinoa*. The viral particles were precipitated from clarified *C. quinoa* infected crude sap. Contaminating proteins were removed by centrifugation on a sucrose cushion and two consecutive sucrose density gradients (Schellenberger et al., 2011a).

- GFLV and ArMV structures: viral structures were obtained by three complementary approaches. A 3D structure of GFLV protein $2C^{CP}$ was obtained by homology modelling reconstruction using the crystal structure of the *Tobacco ringspot virus* (TRSV) CP as template (Schellenberger et al., 2010). Cryo-electron micrographs of GFLV-13 and ArMV-S particles collected under low-dose conditions were recorded, digitized and extracted to reconstruct 3D models of GFLV and ArMV (Schellenberger at al., 2011b). The 3D model of ArMV was further improved to a pseudo-atomic model of ArMV (Lai-Kee-Him et al., 2013). Crystals of two GFLV isolates were produced and X-ray diffraction data were phased by molecular replacement using the 3D GFLV-F13 cryo-EM model to determine the atomic structure of the two GFLV isolates. (Schellenberger et al., 2011b)

- Chimeric virus engineering: substitutions of GFLV sequences by their ArMV counterparts were done by site-directed PCR mutagenesis of the GFLV-F13 RNA2 full-length cDNA clone. Biological properties of chimeric RNA2 were analyzed in protoplasts and *in planta* after co-inoculation with GFLV-F13 RNA1 transcripts. Some mutated CPs were introduced into a GFLV recombinant RNA2 encoding the Enhanced Green Fluorescent Protein to track the virus after transcripts inoculation to herbaceous hosts.

- Nematode transmission assays: Nematode transmission assays relied on a two-step procedure of 8 weeks each. During the acquisition access period (AAP), aviruliferous *Xiphinema spp* were fed on roots of infected source plants, followed by the inoculation access period (IAP), during which infected source plants were replaced by healthy bait plants. The presence of viruses was assessed in roots from bait plants by DAS-ELISA and ImmunoCapture-RT-PCR.

RESULTS AND DISCUSSION

Previous results showed that the CP determines the transmission specificity of the two virus (Andret-Link et al., 2004b, Marmonier et al., 2010). To identify GFLV-CP amino acids involved in the transmission by *X. index*, we hypothesized that candidate residues are likely exposed at the external surface of virions, different between GFLV and ArMV isolates but highly conserved among GFLV isolates. Based on structural GFLV models determined by our structural approaches, 5 surface-exposed regions of GFLV CP, termed R1 to R5 ranging from 4 to 11 residues were identified (Schellenberger et al., 2010). CP mutants were generated by substituting these 5 putative GFLV domains by their ArMV counterparts. The chimeric viruses harbouring R1 and R2 led to a systemic infection *in planta* whereas R3 to R5 were not able to trigger a sytemic infection. Additional site-directed mutagenesis targeting the R4/R5 region allowed to restore a systemic movement of the R4 chimeric virus. Transmission tests revealed that R2 and R4/R5 chimeric viruses were not transmitted by *X. index,* whereas R1 chimeric virus remained transmissible by *X. index*.

Moreover, the characterization of GFLV-TD weakly transmitted, revealed the importance of a single mutation in Gly297Asp (belonging to R5 region) in the transmission process. The crystallographic structure of GFLV-F13 (3 Å) and GFLV-TD (2.7 Å), shows that the loss of transmission of variant originates from the presence of a negatively-charged side chain exposed at the surface of the virion.

The comparisons of the GFLVs atomic structures associated to functionnal approaches allows us to identify 3 viral determinants critical for the virus transmissibilty by *X. index* and to propose a viral pocket-like structure exposed on the surface of the virus particle as the *Xiphinema* Binding Site (XBP). This XBP will be recognized by a specific nematode ligand, not yet identified. Our results will be disccused in regards to the transmission specificity of GFLV and ArMV by their respective nematode vectors and will pave the way for innovative strategies for the fanleaf control.

ACKNOWLEDGEMENTS

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OP 05 - Nanobodies: Getting over the hump of fanleaf degeneration?

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INTRODUCTION

Fanleaf degeneration is one of the most detrimental viral disease of commercial grapevine. Its main causal agent is *Grapevine fanleaf virus* (GFLV), a *Nepovirus* specifically transmitted from grapevine to grapevine by the ectoparasitic nematode *Xiphinema index*. Its broad distribution and prevalence pose serious threat to the sustainability of vineyards worldwide. Structurally, GFLV is an icosahedral virus of 30 nm in diameter with a pseudo $T = 3$ symmetry composed of 60 identical subunits. In recent studies we resolved its atomic structure and identified surface-exposed structural motifs essential for GFLV transmission and movement (Schellenberger et al., 2011)

Current ways to control the virus rely essentially on preventive measures via the distribution of healthy/certified propagative material, decontamination and replanting of infected plots and fallow period of up to 10 years. Such measures are difficult to implement for economical reasons in vineyards of high added value and are clearly insufficient to control the disease in heavily contaminated areas with high vector density. No natural sources of resistance associated to viral disease have been identified in *Vitis* species. Therefore, there is a strong need to engineer GFLV-resistant grapevine varieties to serve as efficient, environment friendly and durable approaches to control fanleaf disease.

Nanobodies (Nbs) are single domain peptides derived from heavy chain only antibodies naturally found in camelids (Muyldermans, 2013). Because of their unique biochemical properties combining monomeric structure, small size and high stability, they have proven to be of outstanding biotechnological interest including as antiviral molecules to neutralize animal viruses. Yet their use in agro-biotechnology is still very confidential. Here we describe the generation of Nbs directed against GFLV and their powerful antiviral activity when constitutively expressed in transgenic *Nicotiana benthamiana*.

MATERIALS AND METHODS

Immunization, cDNA library cloning and Nb screening.

A dromedary (*Camelus dromedarius*) was immunized with purified GFLV-F13. Total RNA was extracted from blood lymphocytes and mRNAs were reverse transcribed to cDNA. The regions encoding the variable fragments of heavy chain antibodies were then amplified with two subsequent PCRs, cloned into a pHEN4 phagemid vector (Ghahroudi et al., 1997) and transformed into *E. coli* TG1 cells. The resulting library was screened by phage display for GFLV-specific binders.

Expression and purification of Nbs from *E. coli*.

GFLV-specific Nbs' coding sequences were sub-cloned into the pHEN6 (Conrath et al., 2001) and the Gateway p0GWA (Busso et al., 2005)expression vectors. Large-scale production was performed in WK6 and BL21(DE3) *E. coli* strains, respectively. Nbs were purified by affinity chromatography followed by size exclusion chromatography.

Transgenic *N.* benthamiana plants.

Nb23 was cloned in frame to the Nterminus of EGFP with a 7 amino-acid linker sequence into a pDONR/Zeo Gateway donor vector and further recombined into a plant expression vector. A control consisting of EGFP cloned in the same vector was included. These constructs were transferred into *Agrobacterium tumefaciens* and used at OD_{600nm} = 0.1 for agro-

transformation of *N.* benthamiana.

Plant inoculation.

Transgenic plants at the 4- to 6- leaf stage were challenged by mechanical inoculation of either purified virions or sap from infected *Chenopodium quinoa* plants and also by nematode inoculation using soil containing viruliferous *X. index*. Systemic infection was assessed by DAS-ELISA 21 days post infection or 16 weeks post-contact with nematodes, respectively.

RESULTS AND DISCUSSION

A pool of 23 Nbs recognizing GFLV particles were retrieved by phage display biopanning of a library generated from a GFLV-immunized dromedary. Following expression in *E. coli* and purification to homogeneity, Nbs' antigen recognition and specificity towards 8 different GFLV isolates from our collection (GFLV-F13, -GHu, -TD, -CO2, -BUChardT60, -BE4.11 and -BE5.19) and one isolate of the closely related *Arabis mosaic virus* (ArMV-S) was tested by DAS-ELISA. One Nb, namely Nb23, was chosen for further characterization. This Nb is GFLV-specific: it recognizes all tested GFLV isolates but is unable to detect ArMV in ELISA. Fusion of Nb23 to EGFP does not interfere with its capacity to specifically bind GFLV *in vitro.* This construct, as well as a control EGFP construct, were then transferred into a plant expression vector and used to stably transform *N. benthamiana* plants. Three homozygous T2 lines, two expressing Nb23:EGFP (lines 23EG16-9 and 23EG38-4) and one expressing EGFP (line EG11-3) were selected. Plants were challenged with either 300 ng or 3 µg of purified virus or with *C. quinoa* infected sap. Apical non-inoculated leaves were analyzed by DAS-ELISA at 21dpi. Both Nb23:EGFP expressing lines showed high level of resistance (line 23EG38-4) or even total immunity (line 23EG16-9) when GFLV-GHu was the challenging virus while all plants were fully susceptible to ArMV-S.

To further characterize the spectrum of resistance, plants were inoculated with crude sap from *C. quinoa* plants infected with the 8 GFLV isolates previously tested for *in vitro* recognition. The results indicate that both homozygous lines expressing Nb23:EGFP present a broad resistance to multiple GFLV isolates.

We also assessed the resistance of homozygous lines to GFLV infection via *X. index*. Again, very strong resistance was observed: GFLV remained completely undetectable in leaves from both 23EG16-9 and 23EG38-4 lines, contrarily to EG11-3 plants which tested 100% ELISA positive.

At very high dose of inoculum, full resistance displayed so far by 23EG16-9 line could be overcome although a majority of plants from Nb23:EGFP lines remained virus free or showed delayed symptoms compared to the control line.

We thus reasoned that Nb23 could exert a selective pressure on GFLV capsid leading to the emergence of escape variants. This was confirmed by sequencing: mutations in the CP were identified that all mapped to the outer surface of the capsid.

One escape variant was further studied by introducing its point mutation into the GFLV-GHu infectious clone (Vigne et al., 2013). As expected, 23EG16-9 plants were fully susceptible to this synthetic escape variant and overcoming the resistance could be correlated to a reduced affinity of Nb23 with the modified capsid. In addition, cryo-electron microscopy studies were performed to precisely map the capsid residues in contact with Nb23. Remarkably, mutations present in escape variants all mapped to the epitope recognized by Nb23. Finally, when assayed for nematode transmission, at least one mutant failed to be transmitted by *X. index*.

All these results demonstrate that Nbs against GFLV perform extremely well as antiviral molecules in plants and overcoming this resistance likely occurs at the detriment of GFLV transmission by its nematode vector suggesting that the epitope recognized by Nb23 plays important function in virus transmission.

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OP 06 - *Grapevine fanleaf virus*: towards the viral protein-protein interaction map and host factors identification.

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INTRODUCTION

Grapevine fanleaf virus (GFLV) is a very damaging nepovirus to vines and wine production throughout the world. It is a bipartite positive-sense RNA virus that replicates on endoplasmic reticulum membranes (Ritzenthaler *et al.*, 2002). Each of the two RNAs encodes a single polyprotein. Polyprotein P1, encoded by RNA1, is processed by the viral proteinase $1D^{Pro}$ into five proteins: protein 1A of unknown function, $1B^{Hel}$ which presents a predicted helicase motif and four to five transmembrane domains, $1C^{VPg}$ which is the viral protein linked to the 5' end of the genome, the already mentioned $1D^{Pro}$ and the polymerase $1E^{Pol}$, which has also recently been described as a symptom determinant in *Nicotiana benthamiana* and *N. clevelandii* (Vigne *et al.*, 2013). Polyprotein P2, translated from the RNA2, gives rise to three proteins: the homing protein $2A^{HP}$ that is involved in RNA2 replication, the movement protein $2B^{MP}$ and the structural protein $2C^{CP}$.

Although GFLV is one of the best characterized grapevine viruses, little information is available on the mechanisms underlying the different steps of the virus life cycle. To gain insights into these mechanisms and especially into replication, a yeast two hybrid approach was used to characterize the viral proteins network. 1A emerged as an interaction hub among viral proteins involved in replication, interacting, among others, with $2A^{HP}$. Bimolecular fluorescence complementation (BiFC) experiments were initiated to confirm the central role of 1A in replication.

In order to better understand how the virus interacts with the host cell during replication, we then used 1A and 2A as baits to screen a cDNA library from *Arabidopsis thaliana.* Host factors interacting with these viral proteins were found to belong to various cellular processes including endomembrane metabolism, RNA modification and transport, translation, proteasomal degradation and autophagy.

MATERIALS AND METHODS

Yeast two hybrid experiments

In an effort to reduce the number of false negatives, we have used the gateway vectors developed by Stellberger and collaborators (2010), which allow both N- and C-terminal fusion of the AD and BD domains of the Gal4 transcription factor to the proteins of interest.

A normalized universal arabidopsis cDNA library in pGAD HA was amplified and used to transform yeast cells bearing either a pGBKT7g-1A or a pGBKCg-2A bait plasmid.

Bimolecular fluorescence complementation (BiFC) experiments

The binary vectors pSITE BiFC nEYFP-N1 and pSITE BiFC nEYFP-C1 for the transient expression of the 173 N-terminal aminoacids of the fluorescent EYFP protein fused at the N- or C-terminus of the viral proteins and the vectors pSITE BiFC cEYFP-N1 and pSITE BiFC cEYFP-C1 allowing the fusion of the 67 last aminoacids of EYFP to the N- or C-terminus of the viral proteins have been described (Martin *et al.*, 2009). *Agrobacterium tumefaciens* GV3101::pMP90 were transformed with the recombinant plasmids and used for infiltration of *Nicotiana benthamiana* plants. Observations were conducted two days post infiltration on a Zeiss LSM700 or LSM780 confocal microscope.

RESULTS AND DISCUSSION

Viral protein-protein interaction map

In order to get insights into the mechanisms underlying the virus life cycle, we undertook to establish the network of interactions between the eight mature proteins described for GFLV. To this end we used the gateway version of the classical yeast two hybrid plasmids allowing the fusion of the activating (AD) and binding (BD) domains of the Gal4 transcription factor to the N-terminus of the protein of interest, as well as the more recent vectors developed by Stellberger and collaborators (2010). These recent vectors fuse the Gal4-AD and -BD domains to the C-terminal end of the protein of interest and therefore, when combined with the classical vectors for N-terminal fusion, they allow four bait-prey combinations (NN, CC,

NC and CN) for each couple of proteins tested. In an exhaustive screen more than 260 combinations were analyzed by mating haploid single transformants and assaying diploïd growth on selective media.

In a second step, detected interactions were confirmed by co-transforming yeast haploid cells with the two plasmids. Altogether this yeast two-hybrid approach identified and confirmed protein 1A as an interaction hub among viral proteins previously identified as important for RNA1 and/or RNA2 replication. In particular the 1A-2A interaction was found in several permutations.

The fluorescence based complementation assay BiFC (Kodama and Hu, 2012) was then used *in planta* to confirm and localize some interactions at the subcellular level.

Host factor screen

To identify host factors important for GFLV life cycle, we screened an *Arabidopsis thaliana* cDNA library in a yeast two-hybrid approach using 1A and 2A proteins as baits. A total of ca. 3.10^6 and 8.10^6 transformants were obtained with 1A and 2A respectively, from which 3000 clones per screen were transferred onto more selective media. Candidate cDNAs were sequenced and subcloned into an expression vector for transient expression *in planta* along with the viral bait protein to check for co-localization. Candidates identified in these two screens are involved in various cellular processes including endomembrane metabolism, RNA modification and transport, translation, proteasomal degradation and autophagy. In particular, several autophagy-related proteins appear to interact with 1A and are present in viral replication complexes during infection. More experiments are underway in order to understand the role of autophagy in viral multiplication.

While the role of autophagy in viral infections is a major topic in animal virology, knowledge on the subject is still very limited in the field of plant viruses.

ACKNOWLEDGEMENTS

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OP 07 - Findings point to long existence of Grapevine fanleaf virus in Iran

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INTRODUCTION

A wide variety of grapevine (*Vitis vinifera* L.) cultivars are grown at 313,315 ha with annual production of 2,795,925 tons in Iran (Anonymous, 2002). The northwest corner of Iran is one of the main production regions. The first report of GFLV in Iran was based on visual symptoms (Ghorbani, 1988). It appears that infected cuttings have contributed to spread of GFLV in Iran as studies on soil samples from vineyards in the northwest has shown no vectoring nematode. All types of GFLV symptoms (Raski et al., 1983) were reported from Iran (Nourinejhad-Zarghani et al., 2013) commonly including fanleaf, yellow mosaic and vein banding.

MATERIALS AND METHODS

Detection by ELISA Generally double antibody sandwich (DAS)-ELISA (Clark and Adams, 1977) and sometimes direct antigen coated (DAC) - ELISA method (Dijkstra and deJager, 1988) were applied.

Detection by RT-PCR We adopted optimizations to amplify different segments of GFLV RNA2. The primers were the crucial part of such optimizations. Reagents were purchased from Fermentas (Lithuania). Reverse transcription was done by the use of oligo $d(T)_{16}$ or a GFLV-specific primer. Initially, previously reported primers (Wetzel et al., 2001) were applied to give 810 bp fragment. New primers were designed after sequences of local isolates were determined to enhance efficiency of the PCRs. As such, GMPF1 and GMPR1 primers were designed for amplification of full length MP gene (1044 bp). Also, we designed GFLV-2048 and GFLV-3559 to amplify the virus full length CP gene. Amplification of the RNA2 was done with 5'-NC/M4 and GFLV2048F/3'NC primer pairs giving 2.2 and 1.65 Kbp segments of the GFLV RNA2, respectively, covering the partial 5'- non coding region, entire $2A^{HP}$ and $2B^{MP}$, and the $2C^{CP}$ with a partial segments from 3'- non-coding region (Nourinejhad-Zarghani et al., 2012).

Cloning and sequencing: Representative PCR-amplified fragments (~20 ng) were ligated into pTZ57R/T (50 ng) (Fermentas, Lithuania) and the resulting recombinant plasmids were transformed into competent *Escherichia coli* cell followed by screening to find respective desired colonies and subsequently subject to sequencing.

Phylogenetic and recombination analyses: Data from sequencing reaction were assembled to determine sequences of different genomic parts of GFLV. Then, the sequences were aligned with counterpart regions of different isolates from Iran and previously reported isolates. The phylogenetic analyses were based on genetic distance or parsimony methods (Felsenstein, 2004). MP Sequences of the isolates from the northwest were aligned with counterpart genomic region of previously reported GFLVs and that of *Arabis mosaic virus* and submitted to RDP3beta41 (Heath et al., 2006).

RESULTS AND DISCUSSION

As a result of three independent surveys during 2003-2007, GFLV was detected by ELISA in collectively 84 out of 346 (24.3%) samples showing that nearly all sampled are as in the northwest were infected by GFLV. However, ELISA detected GFLV in a percentage of samples likely due to a relatively lower sensitivity of ELISA compared to that of RT-PCR. By the help of newly-designed primers different genomic segment of the virus were amplified, cloned and sequenced. By the primers GMPF1 and GMPR1, the full length MP gene (1044 bp) was amplified from 41 of the 86 ELISA-positive samples. Sequence analyses of seven PCR products (MP) revealed up to 17 and 8% divergence between the Iran isolates at NT and deduced AA sequence, respectively. A 1515 bp fragment was obtained for 16 out of 89 samples by the use of GFLV-2048 and GFLV-3559. CP fragment from eight isolates were cloned and the NT sequences determined. Alignment of previously reported GFLV strains/ isolates and ArMV-S showed that new isolates were GFLV.

Also, sequences of near full length RNA2 of four isolates from Iran (accession numbers JQ071374 to JQ071377) were determined (Nourinejhad-Zarghani et al., 2012). RNA2s of GFLV isolates Shir-Amin and Urmia was 3730 NT whereas that

of Takestan and Bonab isolates was 3749 NT, excluding the poly (A) tail. The latter contained the longest $2A^{HP}$ gene among the reported GFLV isolates. Identities of 89–97.6% NT were determined between near full-length RNA2 of the Iranian isolates whereas previously 8.3–84.8% identities were estimated for the other Iranian isolates. GFLV-F13 was the closest isolate to the Iranian isolates at the NT level. At the AA level, there were 90.9–97.9% identities among P2 of the Iranian isolates, whereas 86.3–92.7% between the Iranian isolates and previously reported isolates.

On a parsimonious tree based on the MP coding region, isolates from Iran stood distinct suggesting independent evolution of GFLV in this region. When an alignment of 107 MP sequences was analyzed a total of 12 recombination events were detected in 34 recombinants. Double events were evident in the Iranian isolates Kh29-5, La3-6-1, La3-6-3, LGR12, SI1B and SI1C. Recombination were also documented in other parts of the RNA 2 (Nourinejhad-Zarghani et al., 2012). Based on RNA2, GFLV isolate WAPN173 (USA) was the closest to the isolates from Iran. Compared to the other isolates with available GFLV ORF2 sequences, identity levels of 77.5% and 88.3% were found for the $2B^{MP}$ gene, and at least 83.6% and 92% for the $2C^{CP}$ gene at the NT and AA levels, respectively.

In conclusion these findings points to phylogenetically distinct positions of GFLVs from Iran and recombinant nature of several isolates of the virus from Iran. Considering the region between Caspian and Black seas as the origin of grapevine there is possibility of co-evolution of GFLV with the host plant which requires further investigation.

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OP 08 - Grapevine leafroll and red blotch diseases in Washington State vineyards

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INTRODUCTION

A recent economic impact study estimated that the grape and wine industry in Washington State contributes nearly $8.6 billion to the State's economy and nearly $14.9 billion to the national economy (Stonebridge Research Group™ LLC., 2012). As of 2014, total wine grape acreage in Washington State was estimated to be about 50,000 acres, including new plantings (www.washingtonwine.org; www.wawgg.org). Wine grape growers produced 227,000 tons of wine grapes of all varieties in 2014, an 8 percent increase from 2013 (NAAS, 2015). Statewide expansion of the wine grape acreage has seen a parallel increase in the incidence of virus diseases affecting overall growth and sustainability of Washington State's grape and wine industry. Until recently, grapevine leafroll disease (GLD, Rayapati et al., 2008) was considered as the major virus disease in Washington State vineyards. However, this perception has been revised with the recent report of grapevine red blotch (synonym: Grapevine redleaf) disease (GRBD) in Washington State vineyards (Poojari et al., 2013). Therefore, a survey was conducted during 2014 season to gather data on the distribution of GLD and GRBD.

MATERIALS AND METHODS

Vineyard blocks planted with red- and white-berried wine grape (*Vitis vinifera*) cultivars in six American Viticultural Areas in eastern Washington State were surveyed during August-October 2014 for the presence of GLD and GRBD. In the case of red-berried cultivars, leaf samples were collected from individual grapevines exhibiting typical symptoms of GLD or GRBD and from grapevines suspected for GLD- or GRBD-like symptoms. In white-berried cultivars, leaf samples were collected from grapevines randomly due to the absence of visual symptoms of GLD and GRBD. Samples were extracted according to the protocol described by Rowhani et al. (2000). Sample extracts were tested individually by reverse transcription (RT)-PCR for the presence of *Grapevine leafroll-associated virus 3* (GLRaV-3) using primers designed in-house (Donda et al., unpublished) and by PCR for the presence of GRBaV using primers described in Krenz et al. (2014). The PCR products were resolved in agarose gel electrophoresis to reveal the approximately 540 and 280 base pair (bp) DNA fragments specific to GLRaV-3 and GRBaV, respectively. Appropriate positive and negative controls were used to validate test results. Where ever necessary, virus-specific amplicons from select number of samples were cloned into pCR2.1 (Invitrogen Corp., Carlsbad, CA) and the nucleotide sequence of two clones per amplicon was determined in both orientations. The nucleotide sequences were compared with corresponding sequences available in public databases to confirm the presence of GLRaV-3 and GRBaV. In some cases, the next-generation sequencing was used for identifying candidate virus(es) associated with GLD- and GRBD-like symptoms (Poojari et al., 2013)

RESULTS AND DISCUSSION

Under Washington conditions, symptoms of GLD and GRBD were observed in red-berried cultivars around or soon after *véraison*. Typical symptoms of GLD, consisting of 'green' veins, inter-veinal reddening and downward rolling of leaf margins, and GRBD, consisting of primary veins showing red color and irregular blotches on leaf blades, were observed in some red-berried cultivars. However, these distinct symptoms were not observed in many red-berried cultivars, making it difficult to diagnose GLD and GRBD in vineyards based exclusively on symptoms. Similar to GLD, no visual symptoms of GRBD were observed in white-berried cultivars. Consequently, we tested all samples collected during the survey for GLRaV-3, the most prevalent among the GLRaVs documented in Washington State (Naidu, 2011), and GRBaV by RT-PCR and PCR, respectively.

A total of 546 samples from eleven red-berried cultivars and 30 samples from four white-berried cultivars were tested separately for GLRaV-3 and GRBaV. A combined total of about 82 percent of samples tested positive for GLRaV-3 and GRBaV and about 18 percent of samples tested positive for both viruses. These results indicated single virus infections in the majority of samples tested compared to samples with co-infections of GLRaV-3 and GRBaV. Among the approximately 82 percent samples with single virus infections, about 71 percent and 29 percent samples were positive for GLRaV-3 and GRBaV, respectively. Sequence analysis of virus-specific amplicons of expected size from representative samples

confirmed the data obtained from diagnostic assays. Based on these results, it can be concluded that GLRaV-3 is the most predominant and wide spread virus compared to GRBaV. It should be noted, however, that these results are from one season and we are continuing diagnostic surveys to gain a better understanding of the relative importance of GLD and GRBD to the sustainability of Washington State's wine grape industry.

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OP 09 - Timing of the symptoms influences the management strategies of *Grapevine leafroll-associate virus 3* in New Zealand.

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INTRODUCTION

In New Zealand, *Grapevine leafroll-associated virus 3* (GLRaV-3) is the most widespread and economically damaging virus in vineyards. In an effort to preserve the vineyard asset, visual diagnostics in red berry varieties aim to detect GLRaV-3 infection by looking for characteristic changes to foliage colour and morphology, a technique that supported a successful vine removal (roguing) strategy in South Africa (Pietersen et al. 2013). Thus, in the first part of this study, we sought to determine an optimal time to undertake visual symptom identification for red berry varieties.

Recent GLRaV-3 studies have identified significant genetic variation within the GLRaV-3 populations in New Zealand and worldwide (Maree et al., 2013). To date, little is known about the biological significance of the genetic variation and how these affect the roguing strategy implemented. To understand how the genetic variants of GLRaV-3 affect the grapevine biology, we initiated a study involving the establishment of a comprehensive field trial comparing GLRaV-3 genetic variants from Groups I, VI, and NZ2.

MATERIALS AND METHODS

Commercial block monitoring

The commercial vineyard study block was located in Hawke's Bay, on the east coast of New Zealand's North Island. The block contained 1,243 Cabernet Sauvignon vines, which were planted in 2000. In 2009, 9.9% of the vines were visually identified with GLRaV-3. The vines were visually inspected for symptoms of GLRaV-3 annually thereafter until 2013. Between 2009 and 2011, all vines visually identified with GLRaV-3 were rogued later the same year. However, in 2012, all roguing was deferred *in lieu* of whole block removal in winter (July) 2013. In 2012 and 2013, the block was visited on at least six separate occasions over a period of 75 days, respectively. The first visit of each year, in early February, coincided with (2012) or was just prior to (2013) the onset of berry ripening (véraison). At each visit, symptomatic vines were tagged and the numbers identified were recorded. Visits continued until leaf condition deteriorated to a point where visual assessments were no longer feasible (early- to mid-April).

Field trial of selected GLRaV-3 genetic variants

Over 1000 healthy or GLRaV-3 infected vines were planted in three grape growing regions in New Zealand (Auckland, Hawke's Bay, and Marlborough). These vines included 720 vines green grafted with single infections of GLRaV-3 genetic variants representative of group I, VI, and NZ2, and four grapevine cultivars: Merlot, Pinot noir, Pinot gris, and Sauvignon blanc (i.e. 20 biological replicates for each type of GLRaV-3 variant infection, each of the four cultivars, and each of the three sites). Virus status was confirmed by ELISA and conventional and real-time RT-PCR assays using virus specific primers. To ensure virus inoculum did not carry unwanted grapevine viruses, potential source plants were screened by next generation sequencing (NGS). During the 2014-2015 season, visual symptoms on vines were monitored on at least five occasions over a period of 74 days, ceasing in mid-April.

RESULTS AND DISCUSSION

Commercial block monitoring

In 2012, 99 symptomatic vines were visually identified with GLRaV-3 but they were not rogued. By 53 days post véraison, 93 of these vines could be identified visually. The next year (2013), 94 of these same vines were symptomatic by 20 days post véraison (Figure 1). Thus, 95% of Cabernet Sauvignon vines infected with GLRaV-3 developed foliar symptoms 33 days earlier in the second year of infection. Within-year comparisons between 'old' and 'new' infections were

also assessed (Figure 1). In 2013, the block was visited 11 days before the onset of véraison and while no new symptomatic vines were identified, 27 of the 99 un-rogued vines from 2012 already had foliar symptoms. Six days post véraison, 89 (90%) of the un-rogued vines from 2012 were symptomatic while at the same time, just nine of the 54 (17%) newly identified infections from the total 2013 season had GLRaV-3 symptoms.

From this study, it is clear some vines develop foliar symptoms soon after véraison; in others, symptoms are delayed until post-harvest, and if the symptomology is limited to a few leaves only, some infected vines may not be visually detected. The time of inoculation may explain the temporal variation in symptom appearance between vines. In addition, the presence of genetically divergent GLRaV-3 populations, either as single or mixed infections, could also contribute to differential symptom development.

Field trial of selected GLRaV-3 genetic variants

Preliminary observations from the field trial comparing selected GLRaV-3 genetic variants support the possibility that there are differences in the foliage symptom expression between genetic variants. For all sites, no noticeable symptoms were observed on white grapevine varieties or on healthy control plants. A range of viral symptoms was observed on the foliage of GLRaV-3 infected red vine varieties. Merlot vines (and in most cases, Pinot noir vines) infected with Groups I and VI expressed symptoms earlier than NZ2 infected vines. However, 53 days post véraison, 95-100% of the infected Merlot vines in Hawke's Bay had foliar symptoms that enabled reliable visual identification, independent of the variant (Figure 2). Notably, by the time monitoring ceased, Group I and VI foliage symptoms appeared to be more severe than the vines infected with NZ2.

CONCLUSION

In New Zealand, and probably elsewhere, the timing of visual assessments is likely to be important in determining the incidence of GLRaV-3 infections. Based on the results of our studies, we propose that visual monitoring be undertaken when the greatest likelihood of symptom development exists, which in Hawke's Bay, is at least 50 days post véraison. Ultimately, the observed delay of foliar symptom development in the young Merlot vines infected with GLRaV-3 NZ2 did not confound visual detection when monitoring was undertaken from late-March by an experienced assessor. Assuming assessors are suitably trained, the recommended strategy is for at least two visits per red variety block, which in New Zealand, should be timed for early autumn (mid-March to mid-April).

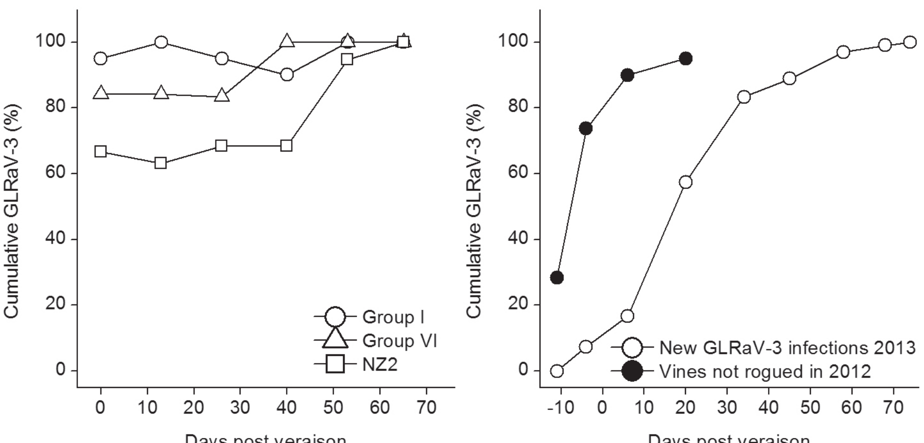

Figure 1. Cumulative percentage changes to Cabernet Sauvignon vines with foliar symptoms of *Grapevine leafroll-associated virus 3* (GLRaV-3) in a Hawke's Bay vineyard during visits in 2013. Difference of symptom expression between the vines already identified symptomatic in 2012 but not rogued (filled circles) and newly identified symptomatic vines (open circles).

Figure 2. Percentage of Merlot vines singly infected with three different Grapevine leafroll-associated virus 3 genotypes that were positively identified by foliar symptoms and were grown in Hawke's Bay in 2015

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OP 10 - Mealybug transmission efficiency of four Grapevine leafroll-associated virus 3 (GLRaV-3) genetic variants

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INTRODUCTION

The rapid spread of Grapevine leafroll disease (GLD) in South African vineyards is of major concern to the wine industry. The incidence of GLRaV-3 variants in local vineyards, their molecular characterisation and transmission efficiency by mealybug vectors play a key role in understanding the biological aspects of GLD spread. *Planococcus ficus* (Hemiptera: Pseudococcidae) is considered the most important vector of GLRaV-3 in South Africa, and it was shown that a single mealybug can transmit GLRaV-3 to a healthy grapevine plant (Douglas and Krüger, 2008). No biological data is currently available on the transmission efficiency of GLRaV-3 variants in vineyards.

To date, six genetic variant groups of GLRaV-3 have been identified world-wide, as well as an additional variant group, group VI-like (Maree et al., 2013). The relative abundance of five GLRaV-3 variants in South African vineyards was determined recently (Jooste et al., 2015). GLRaV-3 variant groups II and VI were the most prevalent as single infections and in combination with each other and other variants (Jooste et al., 2015). However, the importance of the interaction between the mealybug vector and specific GLRaV-3 variants warranted further investigation and thus was the main objective of this study. The transmission efficiency of specific GLRaV-3 variants with *P. ficus* as vector was determined using source plants infected with four characterised GLRaV-3 variants, i.e. group I (represented by isolate 621), group II (represented by isolate 623), group III (represented by PL-20), and group VI (represented by isolate GH11).

MATERIALS AND METHODS

Singly-infected reference plants of four known GLRaV-3 variants (groups I, II, III and VI) were established in a greenhouse. Two additional combinations included *Grapevine virus A* (GVA) with GLRaV-3 group I and group II, respectively. A non-viruliferous culture of *P. ficus* was maintained on butternut (*Cucurbita moschata)*. Virus-free grapevines, *Vitis vinifera* cv. Cabernet franc, were propagated at Vititec (Paarl, Western Cape, South Africa) and served as recipient vines for the experiment. The virus-free status of the plants was confirmed in a GLRaV-3 specific nested RT-PCR (Ling et al., 2001).

To determine the transmission efficiency of the four GLRaV-3 variants as well as the combinations that included GLRaV-3/GVA, single nymphs were carefully transferred with a fine paint brush from infected leaf material after acquisition access periods (AAP) of 48 hours to healthy recipient plants and given an inoculation access period (IAP) of 48 hours. After transmissions the plants were treated with a systemic insecticide. Plants were maintained at 25 ^{o}C, 16:8 L:D and natural humidity. Total RNA was extracted from petioles 8 months after transmissions and tested in a RT-PCR for GLRaV-3 (LR3. HRM4F+R) (Bester et al., 2012) using GoScript and GoTaq (Promega) and nested RT-PCR for detecting GVA (Dovas and Katis, 2003).

Chi-square tests were used to determine transmission differences between GLRaV-3 variants and the combinations with GVA. The significance level was set at 5 %.

RESULTS AND DISCUSSION

A total of 368 single mealybug transmissions were carried out over a three-year period, with 50 to 64 replicates for each GLRaV-3 variant infected source plant (621, 623, PL-20 and GH11). Between 9 and 18% of plants became infected (Figure 1). The number of plants infected did not differ significantly between the GLRaV-3 variant groups (X^2 = 2.14, df = 5, P = 0.828), demonstrating that the four GLRaV-3 variants in single infected vines, or when occurring in combination with GVA, are transmitted equally well under controlled conditions. The results suggest that factors other than the mealybug vector play a role in the prevalence of group I and group VI variants in the field.

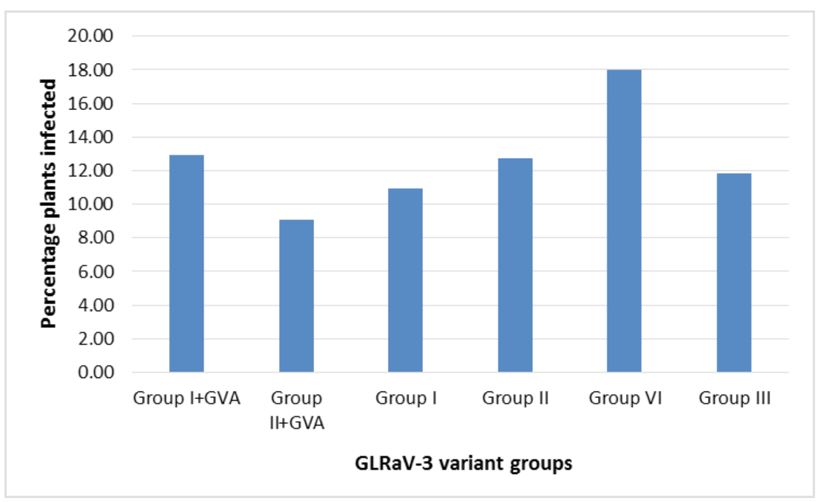

Figure 1. Percentage transmissions of different GLRaV-3 variant groups as well as GLRaV-3/GVA combinations.

Table 1. Number of plants infected from source plants with mixed infections of GLRaV-3 and GVA.

Source plants	GLRaV-3	GVA	GLRaV-3+GVA	Not infected	Total
GLRaV-3 group I/GVA	2 (6%)	16 (47%)	6 (18%)	10 (29%)	34
GLRaV-3 group II/GVA	2 (7%)	18 (60%)	3 (10%)	7 (23%)	30

Mealybugs can transmit GVA from plants with mixed infections without transmitting GLRaV-3 (Table 1). GVA was transmitted more frequently than GLRaV-3. The GLRaV-3 variant had no influence on the number of plants infected with GVA (X^2 = 0.20, df = 1, P = 0.652). With GLRaV-3 group I/GVA and GLRaV-3 group II/GVA as source plants, 65% of and 70% of recipient plants, respectively, became infected with GVA either singly or in combination with GLRaV-3. Blaisdell et al. (2012) also observed that GVA was transmitted more frequently than GLRaV-3 from mixed infected plants and that this can be a major concern for managing GVA spread in vineyards.

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OP 11 - Occurence of *Grapevine leafroll-associated viruses* (GLRaVs) in Aegean Vineyards, Turkey

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INTRODUCTION

Grapevine leafroll-associated viruses (GLRaVs) are one of the most important and harmful viral diseases of the world viticulture production areas. Up till now, twelve GLRaVs have been described as GLRaV-1, GLRaV-2, GLRaV-3, GLRaV-4, GLRaV-5, GLRaV-6, GLRaV-7, GLRaV-8, GLRaV-9, GLRaV-Pr, GLRaV-De and GLRaV-Car. While some of these *Grapevine leafroll-associated viruses* are serologically related but some of them are completely distinct (Martelli et al., 2012). This study was carried out to investigate the occurrence of *Grapevine leafroll-associated viruses* except GLRaV-Car with using serological (DAS-ELISA) and molecular techniques (Real-time PCR) in Aegean viticulture production areas.

MATERIALS AND METHODS

Sample Collection

The survey and collection of samples were conducted between 2011–2012 in Manisa, Denizli, İzmir, Uşak and Aydın provinces which were the major grapevine production areas in Aegean region. Totally, 469.9 hectare areas surveyed and 424 leaf samples were collected randomly in 244 vineyards as mentioned viticulture production areas. Collected samples were labelled, wrapped in plastic and stored at 4°C, until used for the laboratory analysis.

Double-Antibody Sandwich (DAS)-ELISA

All of the collected samples were tested for the presence of GLRaV-1, GLRaV-2, GLRaV-3, GLRaV-4+9, GLRaV-6, GLRaV-7 using DAS-ELISA with commercial kits which were supplied from Bioreba AG (Switzerland). The viruses were tested with reference to the manufacturer's protocol for double antibody sandwich (DAS)-ELISA. Absorbance was determined at 405 nm using Titartek Multiscan Plus MK II ELISA reader (Germany). Samples with absorbance values equal to or greater than two times the average of negative samples were considered positive (infected) according to the Bioreba AG (Switzerland).

Real-Time RT-PCR

Total RNA Extraction

Total RNA was acquired from 200 mg leaf samples using "Zymo ZR Plant RNA MiniPrep™" (Zymo Research Corp., USA) with little modifications on the manufacturer's protocol. Total RNA in 1.5 ml collection tube (approximately 40 µl) was kept at -86°C until used.

Complementary DNA (cDNA) Synthesis

Total RNA were used for complementary DNA (cDNA) synthesis via reverse transcription according to the "First Strand cDNA Synthesis Kit Protocol" (Fermantas, USA). The synthesized cDNA (approximately 20 µl), was stored at -20°C until used.

Real-Time PCR Assays

Real-Time PCR assays were performed with Roche® Real-Time PCR system (LightCycler® Nano Instrument) under appropriate PCR conditions for each viral agent. cDNA mixture were amplified with FastStart Essential DNA Green Master (2x) (Roche®, Germany) which was included Sybr® Green I dye. At the end of the last PCR cycle, melting analyses were performed for eliminate non-specific products like primer dimers. Primers which were used to detect *Grapevine leafroll-associated viruses* (GLRaVs) were obtained for GLRaV-1, GLRaV-2, GLRaV-4, GLRaV-5 (Osman et al. 2007), GLRaV-3 (Osman and Rowhani 2006), GLRaV-6, GLRaV-Pr, GLRaV-De (Maliogka et al. 2008), GLRaV-7 (Engel et al. 2008), GLRaV-8 (Matus et al. 2008) and GLRaV-9 (Alkowni et al. 2004) as referred publications.

RESULTS AND DISCUSSION

DAS-ELISA Results

All of the collected samples were tested for detection of GLRaV-1, GLRaV-2, GLRaV-3, GLRaV-4+9, GLRaV-6 and GLRaV-7 using DAS-ELISA. Forty out of 424 samples (9.43%) were infected at least one viruses according to DAS-ELISA assay. At the end of DAS-ELISA results, GLRaV-3 was found to be most widespread virus with 6.37% infection rate, followed by GLRaV-4+9 (2.12%), GLRaV-2 (0.70%) and GLRaV-1 (0.24 %) respectively. GLRaV-6 and GLRaV-7 were not detected by DAS-ELISA assays.

Real-Time PCR Results

All of the collected samples were tested by Real-Time PCR for detection of GLRaV-1, GLRaV-2, GLRaV-3, GLRaV-4, GLRaV-5, GLRaV-6, GLRaV-7, GLRaV-8, GLRaV-9, GLRaV-Pr and GLRaV-De. 347 out of 424 samples were infected by one (64%) or more (36%) *Grapevine leafroll-associated viruses* according to Real-Time PCR assays. Although some studies about sequence of GLRaV-8 were showed that it's likely of the non-viral origin, also it's a part of grapevine genome according to the hypothesis of reference publications (Bertsch, et al., 2009 and Martelli et al., 2012); GLRaV-8 was found to be the most widespread virus with 75.94% infection rate interestingly that followed by GLRaV-Pr (16.75%), GLRaV-De (12.03%), GLRaV-3 (8.49%), GLRaV-2 (2.83%), GLRaV-4 (2.36%), GLRaV-1 (0.47%), GLRaV-5 and GLRaV-7 (0.24%) respectively. GLRaV-6 was not detected with collected samples likewise DAS-ELISA results. In addition Real-Time PCR, mixed infection of GLRaVs were detected widely (36%) in infected vines. Contrary previous research about GLRaVs occurrence (Akbaş et al. 2007; Akbaş et al. 2009; Çığşar et al. 2002; Yılmaz et al. 1997); GLRaV-1, were not detected widespread as much as in this research.

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OP 12 - Grapevine Leafroll associated virus 1 effects on different Grapevine rootstocks

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INTRODUCTION

Grapevine leafroll disease causes non-uniform maturation of fruits in *Vitis vinifera*, including poor color development in red grape varieties. The disease causes losses of as much as 14-40% with delay of 3 weeks to a month in fruit maturation (Rayapati et al., 2014). To date 4 different viruses, namely *Grapevine leafroll associated virus* (GLRaV) types 1 through -4 have been conclusively shown to be associated with leafroll disease. In the case of GLRaV-4, several distinct leafroll disease-associated virus strains have been identified within the virus species (Martelli et al., 2012).

Our past research has shown that the effects of infection by the GLRaVs depend greatly on the virus as well as the grapevine variety and the rootstocks. In our research, Cabernet franc vines budded onto nine different rootstocks were inoculated with GLRaV-1 from two different sources and planted in the field to evaluate the symptoms, plant growth, yield, berry qualities and berry composition.

MATERIALS AND METHODS

Reference sources of leafroll viruses were established in the Davis Grapevine Collection (Golino, 1992) and regularly updated with newly found viruses and virus strains. Two different isolates of GLRaV-1, LR131 and LR132, used in this experiment were from this collection. Cabernet franc was bench grafted on the following 9 rootstocks: AXR1 (*V. vinifera* 'Aramon' X *V. rupestris* 'Ganzin'), Mgt 101-14 (*V. riperia* X *V. rupestris*), 110R (*V. berlandieri* X *V. rupestris*), 3309C (*V. riparia* X *V. rupestris*), Kober 5BB (*V. berlandieri* X *V. riparia*), 420A Mgt (*V. berlandieri* X *V.riparia*), Freedom (1613C OP seedling X Dog Ridge OP seedling); St. George 15 (*V. rupestris*) infected with *Grapevine rupestris stem pitting associated virus* (GRSPaV) and St. George 18 free from GRSPaV. In 2009 the rootstock portion of these plants was inoculated with two chip buds from each virus source. The inoculated plants were planted in the field which included 15 replicate per treatment per rootstock in three different blocks (5 replicate per block). Symptoms, vine growth, yield, berry composition and color were evaluated in 2014. The symptoms were rated in October from 0 to 4 where 0 represented vines with no symptoms and 4 for vines showing very severe symptoms. For berry composition evaluation, the fruits were harvested when the Brix was approximately 24%. Cases where treatments showed significant differences among rootstocks, leading to significant virus x rootstock interactions, were analyzed and reported by rootstock. JMP® software (version Pro 11, SAS Institute, Inc., Cary, NC) was used for two-way analysis of variance and Tukey's honestly significant difference test at the $p \leq 0.05$ significance level was used to separate means of different treatments.

RESULTS AND DISCUSSION

The data showed that the virus isolate LR132 killed all the Cabernet franc plants propagated on 420A, Freedom, 3309C and 101-14 rootstocks within 1-2 years, therefore, we report the growth, yield and berry evaluations only on the remaining rootstocks for this isolate. None of the rootstocks were killed by LR131 isolate. The real time RT-PCR test results showed that isolate LR132 was co-infected with *Grapevine virus A* (GVA). However, it is not clear yet whether a certain strain of GLRaV-1 is the cause for killing the vines or if the presence of GVA created a synergistic effect that killed the vines. The test also showed that LR131 was co-infected with GRSPaV.

In general, the symptoms rating on the majority of the plants inoculated with isolate LR131 on all 9 rootstocks was 3 (severe). The isolate LR132 was showing more severe symptoms (rating of 3-4) on the surviving rootstocks. Our statistical analyses showed that there was a virus x rootstock interaction and therefore, treatments were analyzed by rootstock. Cane length and pruning weight were significantly lower for all surviving vines on all rootstocks except AXR1 and STG 18, which were not significantly different from healthy. Berry weight, total clusters, and total yield for surviving vines were less uniformly affected by either virus isolate. The only significant reduction in berry weight for either virus treatment occurred in LR131-infected vines on STG.15 and the only significant reduction in total clusters occurred in LR131-infected vines on 3309C. Total yield was significantly reduced in LR131-infected vines on 3309C, 420A and STG.15. Total yield was significantly reduced in surviving LR132-infected vines only on AXR1.

Regarding berry compositions and juice data, no interactions were found between the rootstocks and the virus isolates and the analyses were done independent of the rootstocks. Because there was no berry and juice composition data available for LR132-infected vines grafted on 101.14.1, 3309C, 420A, and Freed 1, only LR131-infected vines were evaluated for these rootstocks. Significant differences were found in ammonia, NOPA, pH, titratable acidity, and YAN compared to healthy vines. In the remaining five rootstocks, LR132-infected vines were most affected showing significant differences in moisture, anthocyanins, potassium, pH, brix and titratable acid. LR131-infected vines were significantly different only in NOPA.

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OP 13 - Detection of Grapevine leafroll-assosiated virus type 3 (GLRaV-3) in Azerbaijan and study of some histopathological changes in leaves of infected plants

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INTRODUCTION

Grapevine (*Vitis vinifera* L.) is widely cultivated fruit species in Azerbaijan as for wine industry as well as fresh and dried fruits. Various viral infections are one of the major factors affecting the development of viticulture and winemaking. Among the virus and virus-like diseases infecting grapevines worldwide, grapevine leafroll disease (GLD) is considered to be the most economically destructive and has been associated with delayed fruit ripening and yield losses of up to 40% (Woodham et al., 1984). GLD symptoms vary within and among vineyards due to several factors including the variety, age of the vineyard, stage of infection, complex of virus's present, viticultural practices, and environmental conditions. Five serologically distinct, phloem limited viruses designated Grapvine leafroll-associated viruses (GLRaV) 1-4 and 7, are associated with GLD (Lee et al., 2009, Martelli et al., 2012). The GLRaV-3 is the predominant virus and widespread in the vineyards of Western Europe, California, Western Washington and South Africa (Tsai et al., 2008, Atallah et al., 2011).

Nevertheless, information on the major grapevine virus diseases and their vectors lacks in Azerbaijan and there are no reports about the incidence and economic impact of GLD on juice and table grapes. The aim of this work was to test the occurrence of GLRaV-3 using different serological tools and the study of some histopathological changes in infected plants. This is the first report of grapevine virus diseases in Azerbaijan.

MATERIALS AND METHODS

During the 2014 growing season, field surveys were conducted in the main grape growing regions of Azerbaijan (Ganja, Samukh, Absheron). Leaf samples with typical leafroll symptoms and symptomless plants were collected from appoximately 36 grapevines of red and white cultivars in late Summer and Autumn (Figure 1). Extarcts were taken from differetent grapes and the initial screening for GLRaV- 3 was carried out by AgriStrip (AgriStrip BIOREBA AG, Switzerland) that allows analyses a large number of samples based on the antigen-antibody reaction as immunochromatographic test. Extracts obtained from leaf samples which showed positive results for GLRaV- 3 were also analyzed with double-antibody sandwich ELISA (DAS-ELISA) according to the general protocol as described (Clark and Adams, 1977) and using commercial specific antibodies (BIOREBA AG, Switzerland). In ELISA tests, all buffers were prepared according to the manufacturer's instructions. Grapevine leaf samples were homogenized (1:5 Wt/Vol) in special extraction buffer "Grapevine" (0.2 M TRIS, pH 8.2) using sterile Bioreba bags and 150 µL volumes of the extracts were incubated overnight at 4°C in the plate previously treated with 150 µL of 1:1000 dilution of GLRaV- 3 IgG in carbonate coating buffer. The virus consentration in samples has been determined using a microplate reader (Biotek, UK) on the basis of optical absorption of enzymatic reaction products at 405 nm in comparison with negative control. Samples with at least three times more values have been considered positive for tested virus disease.

At the same time, some histopathological changes in anatomical structure of the infected and healthy leaves were comparatively studied following the standard methods of anatomy. Samples were killed and fixed in F.A.A. solution (10 ml formalin + 5 ml glacial acetic acid + 50 ml ethyl alcohol 95% + 35 ml distilled water) for 2-3 hours, then dehydrated and cleared in n-butyl alcohol series, and embedded in paraffin wax of 56-58ºC. Cross sections of leaf samples were cut using a rotary microtom and investigated with Fluorescent Biological Microscope DMS-854.

RESULTS AND DISCUSSION

Grapevines in different regions of Azerbaijan were randomly surveyed for presence of Grapevine leafroll associated virus 3 (GLRaV-3).

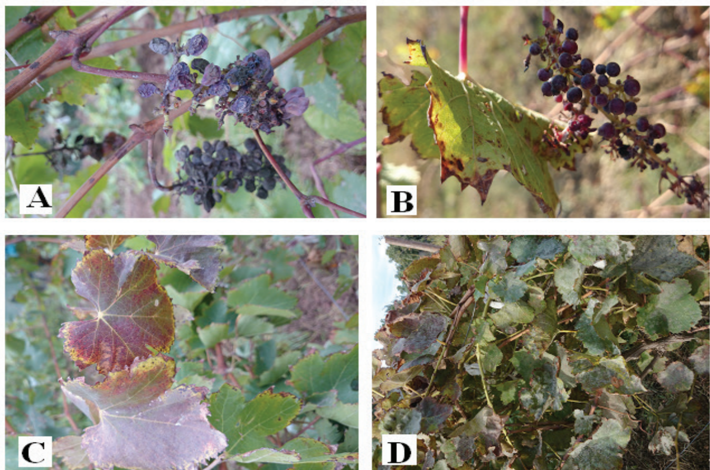

Figure 1. Symptoms of field infections by GLRaV-3 grapevine plants. A - delays fruit ripening, B - symptomatic grapevines with visual appearance of leafroll symptoms, C - symptomatic leaf with rolling of margins and interveinal reddening; D - typical chlorotic vein banding, line pattern and leaf roll.

Symptoms of mosaic, reddish leaf area in red cultivars whereas major veins stay green, typically slight yellowish discoloration of leaves in white cultivars, abnormal leaf size, Z shaped, abnormal shoot development and reduced growth, furthermore characteristically leaves roll were observed in few of the plants (Figure 1). Obtained results by field test showed that both test and control lines become visible with positive extracts (containing GLRaV-3), whereas negative samples produce only the upper control line (Figure 2). Intense coloration was reached within 10-20 min. and the result can be registered. Extracts of leaves of infected plants that were positive in field test (immunostrips) were tested also by DAS-ELISA. Among the collected symptomatic grapevine samples, GLRaV-3 was detected in 58% of the samples by both serologycal tests (immunostrips, DAS-ELISA). Grapevine samples were used for DAS-ELISA tests and values of virus concentration in naturally infected plants showed at table 1.

Table 1. Samples used for ELISA and virus consentration in naturally infected plants.

Plant samples	GLRaV-3	Virus consentration (405 nm)
1. Vine#1	+	2,977
2. Vine#2	+	2, 948
3. Vine#3	-	0,876
4. Vine#4	-	0,526
5. Vine#5	+	2,381
6. Vine#6	+	2,672
7. Vine#7	+	3,076
8. Vine#8	+	2,981
9. Vine#9	-	0,604
10. Vine#10	+	2,887
11. Vine#11	+	3,214
12. Vine#12	+	2,998
13. Vine#13	+	3,065
14. Vine#14	+	3,256
15. Vine#15	+	2,907
16. Vine#16	+	3,672
17. Positive	+	3,558
18. Negative	-	0,106

Figure 2. Detection of GLRaV-3 in infected *Vitis vinifera* L. by serological methods (immunostrips, DAS-ELISA).

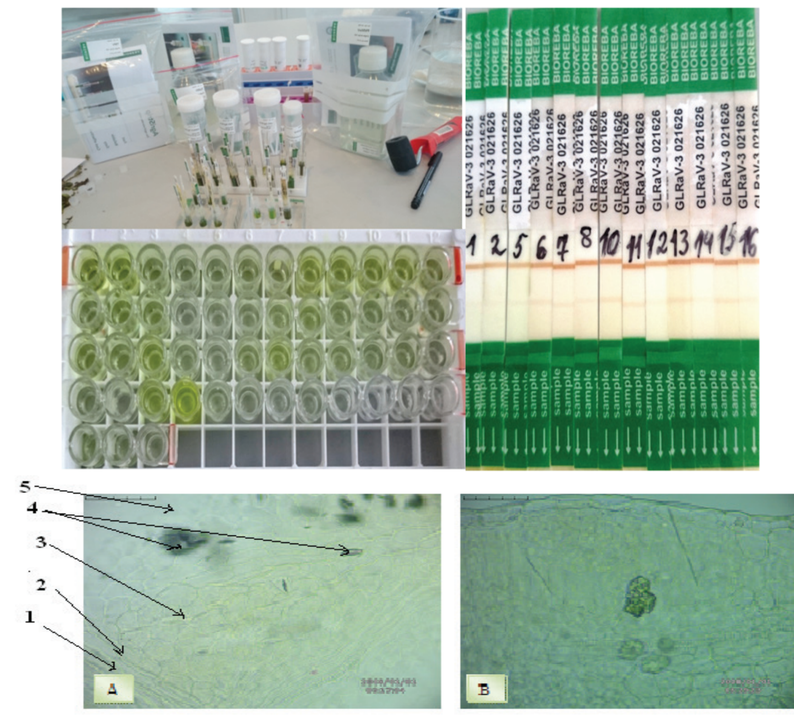

Figure 3. Histopathological changes in anatomical structure of the infected and healthy leaves of grapevine (*Vitis vinifera* L.). A- healthy plant; B- infected plant. 1- lower epidermis; 2-endodermal layer; 3-sponge parenchyma; 4- Salt (calcium-oxalate crystals); 5- Top of epidermis.

At the same time, some ultra-histopathology changes, that occured in the anatomic structure of the infected and healthy leaves were studied comparatively (Figure 3).

Extension of size (20% x 25%) of midvein dimension, increase of midvein vascular bundle dimension, increase of the diameter of xylem vessels, greatly reduction of the leaf petiole and leaf blade thickness, decrease of both spongy and palisade tissues, greatly reduction of the width of cells in the palisade parenchyma, unevenly and poorly lignifed of xylem, reduction of common vesselsand decrease of size cells and extension of xylem rays were observed in the infected leaves as a result of researches with the Light Fluorescence Microscopy

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OP 14 - Serological relationships among GLRaV-4 strains reflects the genetic variability of its coat protein gene

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INTRODUCTION

In the recent history of Grapevine Leafroll Disease, the main criteria used to establish the newly described viral isolates as new species were (in most cases) was serological reaction against previously developed monoclonal antibodies or polyclonal antiserum, and a divergence higher than 10% in the sequence of taxonomic relevant genes (HSP70h and CP). This proliferation of taxonomic entities required a revision of the taxonomy of the GLRaVs, and it was stated than most of the GLRaVs described during the last years (GLRaV-4, -5, -6, -9, -De, -Pr and -Carn) should be considered as isolates of a single species (GLRaV-4) (Martelli et al 2012). Hereinafter this species will be referred to as GLRaV-4sp. Besides the above mentioned, the serological relationships among the GLRaV-4sp groups of isolates (previously known as different species) remain unclear. Gugerli (2009) performed an extensive review of the different serological reagents developed during the 30 years against grapevine leafroll associated viruses. However it is still unclear the significance of the antigenic properties of the CP of the different GLRaV-4sp isolates. In this work, we performed a serological analysis of the CP gene of Argentinean isolates of GLRaV-4sp, and aimed to establish the significance of such serological variability.

MATERIALS AND METHODS

Nineteen plants infected by GLRaV-4sp as determined by PCR, were selected for serological characterization. Viral particles were purified from cortical scrapings of mature canes as described by Savino (1993). The purified virions were resolved over 30 mm wide lanes into a 14%/4% SDS PAGE, and after electroblotting and blocking of the membrane, individual longitudinal strips from each lane were probed with each of five monoclonal antibodies: Mab 36-117 , Mab3-1 , Mab8-2, Mab43-1, Mab3-3 and three polyclonal antiserum: AS GLRaV-5 from Biorad (Hercules, USA), AS GLRaV-6 from Bioreba AG (Switzerland) and AS GLRaV-4 I252-IL. Two additional Mabs (15-5 and 6-3) were analyzed for all the samples. The strips and complete membranes were revealed after incubation with Goat-AntiMouse or Goat-AntiRabbit AP conjugated.

RESULTS AND DISCUSION

The western blot analysis of purified extracts from nineteen GLRaV-4sp infected plants and a virus free accession revealed a variable specificity from the different Mabs and AS used. Two of the three AS used (AS-GLRaV-5 and AS-252-IL) showed a nonspecific reaction, as several bands were observed in all the analyzed samples, even the virus free Chardonnay. However it was clearly identified the GLRaV-4sp CP band. The three AS reacted with the GLRaV-4sp CP of the eighteen extracts analyzed but the sample Sangiovesse Fiano, which only reacted faintly with AS-I252-IL. Two Mabs (6-3 and 15-5) didn't react with any sample. The five remaining Mabs showed variable reaction with the tested samples, from clear to faint bands. The results are resumed in Table 1. The results of the epitope prediction analysis previously conducted revealed a considerably high probability of the occurrence of a linear B-cell epitope in the N-terminal region of CP, consistent with previous observations (Maliogka et al. 2008; Esteves et al. 2012). Considering that most serological reagents available for characterizing the GLRaV-4sp are monoclonal antibodies (Gugerli 2009) and some of them possess good reactivity against the denatured CP in Western blots, it is highly probable that they are directed against a linear epitope. Moreover, considering that viruses were applied in the native form during immunization, these epitopes may be located on the virion surface. Considering that the most immunogenic region may be a linear epitope (the most variable region of the protein), the monoclonal antibodies targeting these epitopes will not be useful for taxonomic assignation at the species level. This asseveration is made considering that the identity level found in this study for GLRaV-5 in the N-terminal region ranges from 29 to 47% and for GLRaV-6 ranges from 29 to 47%. However, these antibodies are very useful for strain discrimination. Conversely, the antibodies present in the commercially available reagent set for GLRaV-4-9 (Besse et al. 2009) appears to target a conformational epitope (as they are nonreactive against the denatured CP in western blots) that is highly conserved and probably located in the C-terminal region of CP. Since no systems are available to predict conformational epitopes from the primary structure of the proteins, this issue remains unresolved.

Table 1. Serological reactivity of extracts from GLD affected vines against Mabs and PAS

The reactivity was determined as positive (+) when a clear intense band was observed, and faint (*f*) when a band of low intensity was recorded .

Grapevine source	Mab36-117	Mab3-1	Mab8-2	Mab43-1	Mab3-3	Mab6-3	Mab15-5	PAS GLRaV-5	PAS GLRaV-5	PAS I-252-IL
Aspirant Bouchet	+	-	-	-	*f*	-	-	+	+	+
Cabernet Sauvignon M1	+	*f*	-	-	*f*	-	-	+	+	+
Foster WS	+	-	-	-	*f*	-	-	+	+	+
Cabernet Sauvignon D33	+	-	-	-	+	-	-	+	+	+
Carmenere	+	-	-	-	+	-	-	+	+	+
Plavai	+	*f*	*f*	*f*	+	-	-	+	+	+
Unidentified cv.	+	-	-	-	+	-	-	+	+	+
Gamay de Freaux	+	-	-	-	+	-	-	+	+	+
Sacy	+	-	-	-	+	-	-	+	+	+
Masse Camp	*f*	*f*	+	*f*	*f*	-	-	+	+	+
Cabernet Sauvignon LR50	-	-	+	-	-	-	-	+	+	+
Colgadera	-	-	+	-	-	-	-	+	+	+
Verduzco Friulano	-	-	+	-	-	-	-	+	+	+
Shuyler	-	-	+	+	+	-	-	+	+	+
Escobera	-	-	+	+	+	-	-	+	+	+
Chardonnay (Healthy)	-	-	-	-	-	-	-	-	-	-
Sangiovesse Fiano	-	-	-	-	-	-	-	-	-	f
Tamares	-	-	-	-	-	-	-	+	+	+
Tockai	-	-	-	-	-	-	-	+	+	+
Teneron	-	-	-	-	-	-	-	+	+	+

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OP 15 - GLRaV-3: diversity, detection and quantitation

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INTRODUCTION

Grapevine leafroll-associated virus 3 (GLRaV-3) is the most widely studied grapevine virus, yet comparatively few genomes have been fully sequenced. The result of this knowledge gap is that studies investigating evolutionary history, diversity and recombination are severely hampered and show very slow progress. In 2012 we identified a divergent variant of GLRaV-3 in a *Vitis vinifera* cv. Cabernet Sauvignon plant designated GH24, using a metagenomic next-generation sequencing (NGS) approach using extracted double-stranded RNA. The low sequence homology of the *de novo* assembled contigs to other known GLRaV-3 isolates clearly identified isolate GH24 as yet another genetic variant of GLRaV-3 and the most distantly related variant identified to date. We set out to characterize this new variant by confirming its full genome with Sanger sequencing and determine its phylogenetic position relative to the rest of the GLRaV-3 genetic variants using all the sequence data available on Genbank (Maree et al., 2015). Concurrently, we also augmented our detection assays to ensure that they can detect and quantitate all known South African GLRaV-3 variants, including GH24 (Bester et al., 2014).

MATERIALS AND METHODS

Sequencing of GH24: Double-stranded RNA was extracted from plant GH24 phloem using an adapted cellulose affinity chromatography method (Burger and Maree, 2015). Illumina NGS data was generated and bioinformatically analysed using a combination of commercial and free software to generate a draft genome. Direct Sanger sequencing of overlapping amplicons was used for validation.

Phylogenetic analysis: A supermatrix was constructed that included a total of 819 GLRaV-3 accessions, represented by sequences of differing lengths, obtained from GenBank. At the time of the analysis, only 13 complete GLRaV-3 genome sequences were available. A thorough phylogenetic analysis was conducted with RAxML (Stamatakis, 2006) and PAUP* (Swofford, 2003) on genome regions devoid of potential recombination sites as predicted by RDP4 (Martin et al., 2010). To estimate node ages (i.e. the ages of common ancestors of different GLRaV-3 strains) and to make an estimation of the rooting of the GLRaV-3 tree that is independent of comparison to the (genetically rather distant) GLRaV-1 outgroup, we performed Bayesian phylogenetic inference and molecular dating using BEAST with the outgroup removed (Drummond and Rambaut, 2007).

Detection and quantitation: Three conserved genome regions were identified through multiple alignments of whole genomes. Primer sets were developed that could detect and quantitate all variants of GLRaV-3 for which sequence data was available. These primer sets were applied in a spatial distribution experiment, evaluating the relative concentration of GLRaV-3 variant group II and VI along the length of a cane. For more detail on MATERIALS AND METHODS refer to Maree et al., 2015 and Bester et al., 2014.

RESULTS AND DISCUSSION

De novo assembly of NGS data yielded contigs covering 84% of the GLRaV-3 genome. Using these contigs a draft sequence was constructed and used to design primers for direct Sanger sequencing of amplicons. The complete genome of GLRaV-3 isolate GH24 (KM058745) was found to be 18493nt long with a typical GLRaV-3 genome organisation, however, no ORF 2 could be identified. The overall sequence similarity to other GLRaV-3 whole genomes is <66%, which is low compared to values of >85% observed between isolates of group I, group II, and group III. GH24 does show high sequence similarity (99%) to partial genome sequences of isolate CB19 (USA), isolate Tempr (Italy), and isolate GTG10 (South Africa). These isolates are likely representatives of the same genetic variant group, which has thus already been detected across the world.

Phylogenetic analysis of the supermatrix that includes the 392 sequences of 602nt in length or more is presented in figure 1. The analysis could resolve currently recognised variants, as well as two clades representing new variant groups

VII (includes isolate GH24) and VIII. Four more inclusive clades are also indicated, defined as supergroups A to D. From the sequence variation it is apparent that GH24 is not closely related to any other GLRaV-3 clade and on that basis can best be regarded as representing a separate supergroup. The significant genetic distance between isolates in supergroup B and between GLRaV-3 supergroups in general, raises some questions about the taxonomic boundaries of GLRaV-3 as a virus species.Three SYBR green real-time RT-PCR assays were developed to detect and quantitate GLRaV-3 in infected vines. Three genomic regions (ORF1a, coat protein and 3'UTR) were targeted to quantitate GLRaV-3 relative to three stably expressed reference genes (actin, GAPDH and α-tubulin). These assays can detect all known variant groups of GLRaV-3 in South Africa with equal efficiency (Group I, II, III, VI and VII). Interestingly, no link could be established between the virus concentration ratios (VCR) of the assays targeting the three genomic regions and subgenomic RNA (sgRNA) expression. However, a significant lower virus concentration ratio for plants infected with variant group VI compared to variant group II was observed for all three assays (Fig 2). Significant higher VCRs were detected in the growth tip for both variant groups. The quantitation of viral genomic regions under different conditions can contribute to our understanding of the disease aetiology.

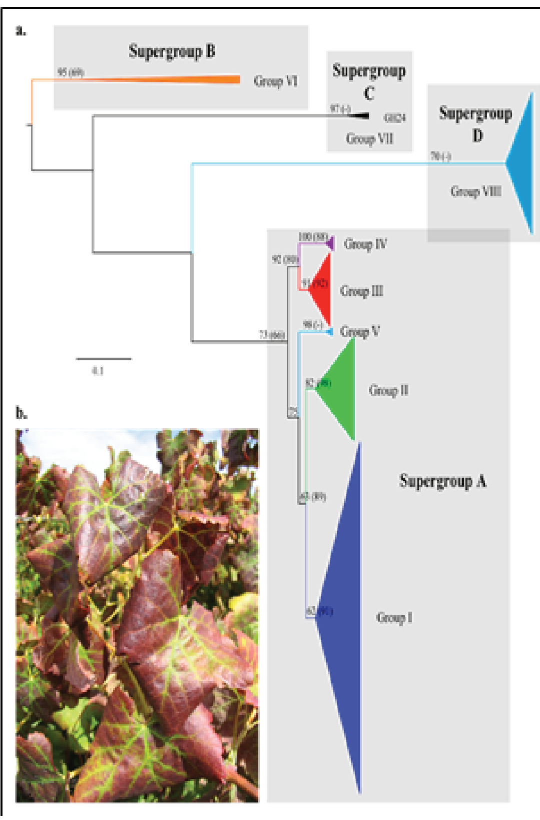

Figure 1: Summary of RAxML phylogenetic analysis. (a) 392 GLRaV-3 sequences of 602nt or more in length were used for the analysis. The tree is rooted, but the outgroups have been removed for ease of presentation; the scale indicates branch lengths in substitutions per site. Values at nodes are ML bootstrap support: first, given this tree, thereafter (within parentheses) support for the equivalent clade given the analysis including sequences of 4761nt or more in length. Groups and supergroups proposed here are indicated. (b) A grapevine (Vitis vinifera cv. Cabernet Sauvignon) showing typical symptoms of Grapevine leafroll disease (Maree et al., 2015).

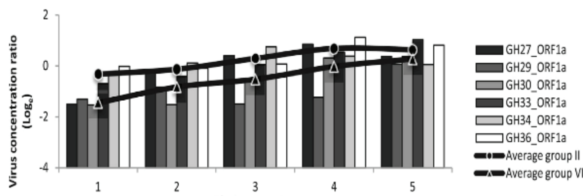

Figure 2: Natural logarithm of the virus concentration ratio (VCR) calculated for each plant segment measured with the three GLRaV-3 assays for ORF1a. GLRaV-3 group VI isolates are GH27, GH29 and GH30. GLRaV-3 group II isolates are GH33, GH34 and GH36. (Bester et al., 2014).

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OP 16 - Comparative sequence analysis of partial coat protein genes of different *Grapevine leafroll-associated virus-1 isolates*

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INTRODUCTION

Leafroll is one of the most important virus diseases of grapevine and the disease was described in the 19th century in Europe. It occurs in most of major grape-growing region of the world and among the virus and virus-like diseases infecting grapevines worldwide, grapevine leafroll disease is considered to be the most economically destructive. GLRaV-1 (the genus *Ampelovirus,* the family *Closteroviridae*) is one of the most important and widespread agent associated with the leafroll disease of grapevines (Martelli et al., 2006). Although grapevine leafroll disease can affect all *Vitis vinifera* cultivars, hybrids, and rootstocks, a new possible host (pomegranate) is detected in Turkey (Çağlayan et al., 2014). The aim of this study was to compare partial coat protein gene sequences of Turkish GLRaV-1 grapevine and pomegranate isolates with worldwide grapevine isolates.

MATERIALS AND METHODS

65 suspicious leaf samples of grapevine and 30 pomegranate samples were collected from different locations of Turkey. The RNA extractions were done using a commercial kit (Qiagen, RNeasy Plant Mini Kit). cDNA was synthesized from total RNAs using random primers with the Super Script Choice System (Invitrogen, USA) and amplified by PCR with specific primers designed in the CP gene of GLRaV-1 isolates from grapevine (Alabi et al., 2011). The obtained PCR products were directly sequenced for both directions. Multiple sequence alignments were performed with the algorithm CLUSTAL W (Larkin et al., 2007). The neighbor-joining method was used to reconstruct phylogenetic trees with nucleotide identity distances implemented in the program MEGA 5.05 (Tamura et al., 2011). Three GLRaV-1 isolates from pomegranates (Çağlayan et al., 2014) and nine isolates from grapevines from Turkey were used for comparative sequence analysis. The deposited GLRaV-1 CP sequences in GenBank (NCBI) from California, Portugal, China, Iran, Poland, India and South Africa were used for the analysis.

RESULTS AND DISCUSSION

Sequence analyses of the GLRaV-1 pomegranate isolates showed at least 91% identity at the nucleotide level with GLRaV-1 isolates from grapevines and the highest identity (94%) was obtained with Californian isolates (JF811845.1) (Fig. 1). Although the isolates were collected from different locations, the phylogenetic analysis showed that there is no clear correlation between collection regions and isolates. The isolates were clustered together with Californian, Indian, Chinese and South African isolates. These results indicate that the partial CP gene of different GLRaV1 isolates from grapevine and pomegranates is highly divergent.

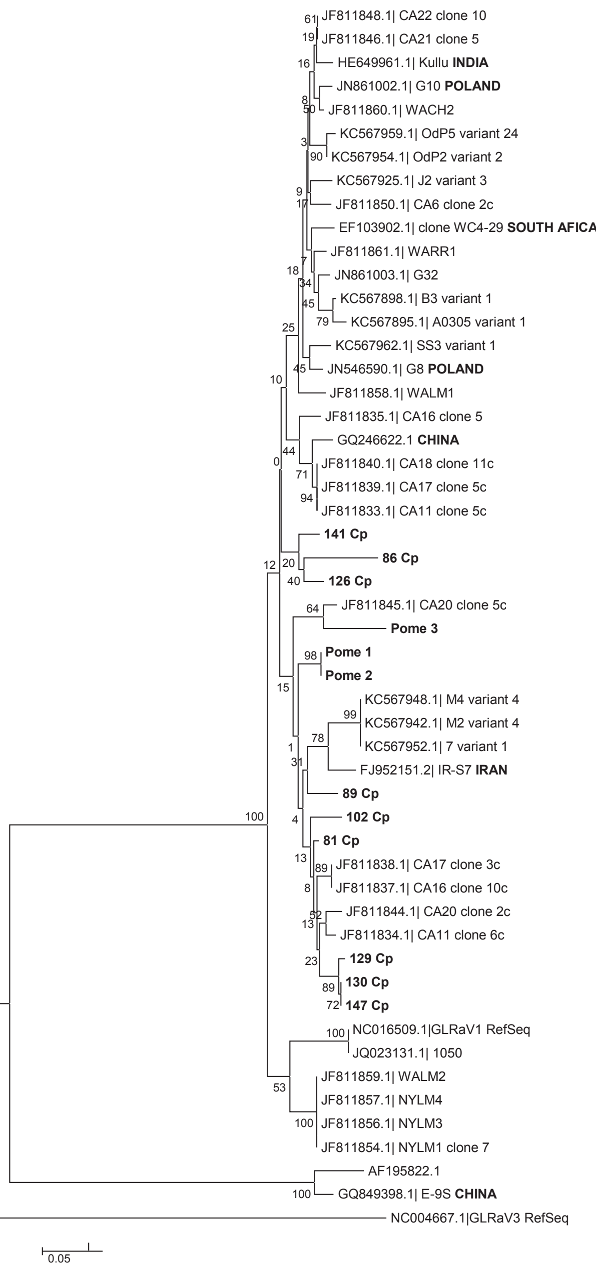

Figure 1. Phylogenetic tree constructed with coat protein sequences of *Grapevine leafroll-associated virus* 1 (GLRaV-1) isolates worldwide. Numbers at the nodes represent the percentages determined by the bootstrap analysis with 500 replicates and only values higher than 50% are shown. GLRaV1 sequences deposited in GenBank (NCBI) were used for the analysis (JN861002-003- JN546590 Poland, HE649961 India, GQ126622- GQ849398 China, FJ952151 Iran, AF195822 Australia, JF811… and HQ833… California, KC567… Portugal isolates) and the obtained isolates under this study are highlighted in bold. *Grapevine leafroll-associated virus-3* (GLRaV3-RefSeq-Acc. no. NC004667.1) was used as out-group control.

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OP 17 - Monitoring in Israel of Grape leafroll disease in new plantings originating from imported plant material.

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INTRODUCTION

Grapevine leafroll disease (GLD) in Israel is mostly caused by grapevine leafroll associated virus 3 (GLRaV3). The disease causes heavy damage to growers and wineries because of its effect on grape and wine quality. Grapevine planting material in Israel is under governmental supervision and nurseries are supposed to get their cuttings from registered mother blocks. In 2007, due to rapid spread of GLRaV3 in the main mother plot a temporary regulation allowed the importation of planting material from France, after close survey and molecular tests of specific registered plots. This regulation lasted for three years (2009-2011) during which ca. 2.5 milion plantlets were made from imported plant material in local nurseries. High awareness to the potential problems with GLRD, triggered many of the growers to monitor for symptom appearance and indeed, in 2012 suspected looking vines began to appear. Most of those symptomatic vines resulted negative to GLRaV3 using the primer LC1 and LC2 (Osman et al., 2007) which are successfully detecting the virus in old plantings in Israel. In the present work we used GIS tools to understand the disease spread in several vineyard plots, found several GLRaV3 variants (new to Israel) in the new plantings and looked at the effects of the different variants on fruit composition and yield.

MATERIALS AND METHODS

Nine vineyard plots (6 Cabernet sauvignon and 3 Merlot), 1-2 hectars each were included in the survey. All were rigorously treated against *Planoccocus ficus*, by applying Imidacloprid (2 ml./vine), mating disruption (620 patches/hectar. Suterra LTD) or both. The plots were surveyed for symptoms every autumn by two experienced people, walking slowly on both sides of the rows. Vines were marked as suspected if typical reddening appeared between the veins. Canes or leaves of sample vines from each plot were taken for molecular diagnostics. The symptomatic vines were pulled out in most of the plots. The relative position of vines showing leafroll symptoms in each year was recorded using the row and vine numbers. A vineyard spatial layer (1142 points) was created in an ArcGIS 9.3 (ESRI Ltd, Manuka, ACT, Australia) using extensions from GeoTools (http://www.ian-ko.com) and used to calculate the distribution pattern of the symptomatic vines.

RNA extraction was done from Leaf petioles that were collected from the first leaf above the cluster, according to chang and Cairney 1993 with modifications. 6 leaves 3 from each side of the row were used. 2.5 µg of total RNA was used for cDNA synthesis and PCR for detection of GLRaV3 was done by using different primers according to primers list.

Vine performance was tested at harvest. Symptomatic molecular diagnosed vines and the same number of healthy looking vines were marked. Berries (100) were picked from each vine. Half were used for must analysis (Brix, pH and TA) and half for color measurement after extraction with acidic ethanol.

RESULTS

Symptoms appearance:

Big differences were seen in infection level between the plots. They could partly be attributed to the planting material (CS clone 15 more symptomatic then clone 338, table 1) and partly to the neighboring vineyards. In most cases symptomatic vines appeared randomly in the plot and only in one vineyard a gradient in symptom incidence could be seen from the edge adjacent to an old infected vineyard.

Table 1:

	Vineyard	stock	clone	Symptomatic vines (2011-2014)	Neighboring vineyards
Cabernet sauvignon	YFT	Richter 110	338	16.32%	North
	GSH	SO4	15	4.18%	West
	GSH	Paulsen	338	2.33%	West
	RMGS	Richter 110	169	1.01%	None
	ELR			0.99%	North, east
	MAL			0.80%	None
	YON	Richter 110	169	0.40%	East
	YON	Paulsen	338	0.50%	None
Merlot	YFT	SO4	181	2.66%	South
	GSH	Paulsen	181	2.10%	South
	KST			0.30%	None

Molecular typing.

While all symptomatic vines sampled from old vineyards, sourced from "Israeli" planting material were found positive for GLRaV3 using primers LC1 F & LC2 R (Osman et al., 2007), very few of the symptomatic vines originating from "new" plant material showed positive PCR results with those primers. Primers LR3 7138R & LR3 6995F, developed by Bester (2014) reviled some more positives and a LC1 with a primer aimed at an inner position compared to LC2, gave a few more. Still, even after using all those sequences ca. 20% of the symptomatic vines remained negative to GLRaV3, as well as to other known grapevine viruses.

DISCUSSION

Though GLRaV3 is found in all grape growing areas of the world the different variants are not. This work emphasis the importance of multi-testing imported planting material with all possible primers and even further, the importance of indexing.

ACKNOWLEDGMENT

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OP 18 - *In vitro* expression and purification of coat protein gene of *Grapevine leafroll-associated virus 3* (GLRaV-3) from grapevine and development of immunodiagnostics for its detection

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INTRODUCTION

India has been a leading country in grape productivity for many years and with the current progression of wine making, the grapevine cultivation in India has been increasing at a very high rate (Adsule et al. 2011). Recent studies have confirmed the presence of grapevine leafroll disease (GLD) in the vineyards of India (Kumar et al. 2012a, 2012b, 2012c; Kumar et al. 2013). This disease is graft transmissible and can be caused by eleven serologically distinct viruses belonging to the family *Closteroviridae* (Martelli et al. 2012). Out of these, *Grapevine leafroll-associated virus 3* (GLRV-3) is the most common and most threatening agent at global level (Sharma et al. 2011; Fuchs et al. 2009). Being as graft transmissible, the best way to check the spread of GLD is to follow the management strategies meant for first line of defence i.e. using the virus free propagating materials at the time of vineyard establishment or replacement of affected vines (Rayapati et al. 2008). For producing GLD free propagating materials it is necessary to screen the large number of vines at nursery stage. Enzyme linked immunosorbant assay (ELISA) has been the most widely used method for screening of viruses (Gugerli, 2009). For ELISA there is a need to have antibodies/antisera. Conventional method of antisera production based on virus purification is problematic because of mixed infection, location of virus particles, low virus titre and presence of inhibitory compounds. Recombinant DNA technology based on the cloning and expression of virus specific genes has provided the way for purification has now made it possible for *in vitro* expression of a specific gene in bacterial or yeast system and use the purified expressed proteins as antigens for antibody production which can be further used for screening of planting materials through ELISA.

MATERIALS AND METHODS

A previously pGEMT cloned CP gene of Nashik isolate (from cultivar Cabernet Souvignon) of GLRaV-3 (Accession no. JN616386) (Kumar et al. 2012a) and maintained in DH5α strain of *Eschericia coli* was selected for expression and subsequent production of polyclonal antisera. This gene was cloned in the pET 28a (+) expression vector using *Eco*RI and *Xho*I (MBI FermentasInc, Germany) restriction enzymes. Recombinant plasmid pET-GLRaV-3-CP was finally transformed to BL21 (DE3) strain of *E. coli* following standard protocol (Sambrook and Russel, 2001). BL21 cells were cultured and induced by addition of isopropyle-1-thio-β-D-galactose (IPTG). The protein was purified from insoluble fraction by Ni^{2+} metal affinity chromatography, purified protein reacted positively in western blotting with commercial anti GLRaV-3 polyclonal antiserum (Bioreba, Switzerland) and hence used as immunogen in two different quantities of 500 µg & 100 µg for the production of polyclonal antisera in New Zealand white rabbits. Primary antisera obtained from rabbits injected with proteins were used in two fold serial dilutions for their assessment through DAS-ELISA (Clark and Adams, 1977) using commercial GLRaV-3 specific conjugate linked with alkaline phosphatase (Bioreba, Switzerland). Globulin fraction (IgG) was purified from polyclonal antiserum obtained from a rabbit injected with 500 µg proteins following protein A-sepharose immobilized Ni^{+2} affinity chromatography. Conjugate was prepared using one step glutaraldehyde method of Clark and Adams (1977). 200 µl of 2500 units of enzyme alkaline phosphatase (Sigma) was mixed with 2 ml of purified IgG (1 mg/ml). *In house* generated primary polyclonal antisera and GLRaV-3 specific ALP-linked-IgG conjugate (secondary antibodies/antiserum) were evaluated together through DAS-ELISA.

RESULTS AND DISCUSSION

The CP gene of GLRaV-3 was over expressed as fusion protein with 6 histone amino acid (6H) repeats at its N terminal (HisTag). In SDS-PAGE analysis of expressed coat protein a band of molecular mass corresponding to ~ 43 kDa, an expected value of fusion coat protein (including the mol. wt. of 6 His-tag), was observed (Fig.1). CP purified from insoluble fraction was confirmed in western blotting. Crude antiserum obtained from each of the three rabbits immunized with two different quantities (two rabbits were immunized in duplication at 500 µg while a third rabbit was immunized with 100 µg) of purified CP of GLRaV-3 reacted well in 2 min of enzyme substrate reaction as compared to the pre-bleeds or pre-immune sera of the respective rabbits. Polyclonal antisera obtained from differentially immunized rabbits reacted specifically in DAS-ELISA and western blotting using commercial conjugate (Bioreba, Switzerland). The obtained purified globulin fraction (IgG) was measured at A_{280} and concentration was found to be as 5.41 mg/ml. The purified IgG reacted against specific antigen

(purified coat protein) by indirect ELISA. Around 1 ml of ALP linked IgG was obtained which reacted specifically to purified CP of GLRaV-3 in direct ELISA.

Primary antisera and ALP conjugated IgG generated in the study could detect GLRaV-3 in the infected sample up to 1:8,000 dilutions of primary antiserum and 1:10,000 dilutions of conjugate Using lesser amount of protein for immunization of rabbit (100 µg) was sufficient enough to detect the virus in DAS-ELISA and western blotting. Antibodies developed against the recombinantly expressed CP in the present investigation specifically and sensitively detected the antigen in both kinds of assays. To the best of our knowledge, it is the first study wherein the CP of GLRaV-3 was cloned in pET 28a (+) vector, having many advantages over others, and further subjected to purification. The indigenously developed immunereagents will provide a cost-effective way of managing grapevine leafroll disease. In addition to provide a helping hand in large scale screening of grapevine propagating materials to produce GLRaV-3 free grapevines, the developed immunoreagents will also be instrumental in the virus-specific quarantine certification programmes of the country.

ACKNOWLEDGEMENTS

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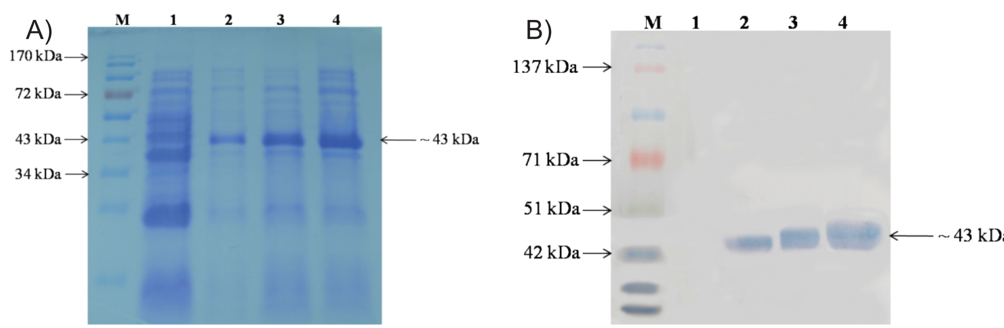

Figure 1: Polyacrylamide gel electrophoresis (PAGE) showing *in-vitro* expression of coat protein (CP) of *Grapevine leafroll-associated virus 3 (GLRaV-3)* cloned in pET 28a (+) vector and transformed into *E. coli* strain BL 21 (DE3) expression system (A). Western blotting analysis of *in vitro* expressed CP of GLRaV-3 with commercial GLRaV-3 specific primary antiserum and GLRaV-3 specific conjugate (both from Bioreba, Switzerland). Lane M – Pageruler prestained protein ladder (Fermentas lifesciences, USA) in Fig. A and Puregene prestained protein ladder from *genetix* Biotech, New Delhi, India in Fig. B; lanes 1 – un-induced; 2, 3 and 4 – 1 h, 2 h, 3 h post induction.

OP 19 - Occurrence and characterization of *Grapevine leafroll-associated virus 4* in Indian Vineyards

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INTRODUCTION

Grapevine is an important crop of India contributing the highest foreign exchange among the fruit crops of the country (Adsule et.al. 2011). It is affected by many diseases caused by various pathogens including viruses (Shaughnessy 2012). More than 55 taxonomically distinct viruses infects grapevine (Martelli, 2003). Grapevine leafroll disease (GLRD) is a one of the most economically important viral disease, exhibiting significant level of threat to the crop productivity as well as grapevine industry. It accounts for 62% yield loss in global grape production (Little et al.2006, Coetzee et al., 2010). GLD has been caused by eleven distinct viruses (Martelli et al., 2012). Out of these, two viruses namely*, Grapevine leafroll -associated virus* 1 (GLRaV-1) and *Grapeine leafroll -associated virus* 3 (GLRaV-3) have been reported from Indian vineyards in recent years (Kumar et al. 2012a, 2012b Kumar et al. 2013). There was the possibility of occurrence of other viruses as well in Indian vineyards. Keeping this in view, grapevine samples from different gape growing regions of India were screened for other viruses; and find the association of *Grapevine leafroll-associated virus 4* (GLRaV-4). GLRaV-4 has been characterized on the basis of taxonomically relevant gene i.e. coat protein (CP). Based on CP genes phylogenetic relationship of GLRaV-4 with other ampeloviruses has been established and potential intraspecies recombination event have been identified.

MATERIALS AND METHODS

Surveys were conducted during the periods of 2012-13, 2013-14 and 2014-15 in different grape growing regions of India and GLD specific symptomatic leaves were collected. The collected samples were tested for the presence GLRaV 4-9 viruses through double antibody sandwich -enzyme linked immunosorbant assay (DAS-ELISA) using commercially available GLRaV 4-9 antisera containing mixture of monoclonal antibodies (Bioreba, Switzerland) following manufacturer's protocol. ELISA positive samples were subjected to two steps PCR for specific detection of GLRaV-4,-5,-6, and -9. In case of GLRaV-4, primer pair CPF' ATGGCAAATCTCGGTGGTAACG, CPR' TCATCTCCTGTTGCCCAAGAAAAT was designed from available CP sequences. RNA was isolated from the infected samples (Sigma Aldrich kit, UK). Complementary DNA (cDNA) was synthesized and cDNAs were amplified with CP specific primers. The specific amplicons of GLRaV-4 CP were purified (QIAGEN GmbH, Hilden, Germany) and cloned into TA cloning vector (RBC,UK).The positive transformants were confirmed by colony PCR and two clones of each isolate were sequenced in both direction. The sequences were analysed using BLAST (www.ncbi.nlm.nih.gov/), CLUSTAL W multiple alignments of *BioEdit* version 7.9.1 software. Phylogenetic analysis was done in MEGA 6 (Tamura et al. 2013) and potential recombination was identified in RDP4 programme (Martin et al. 2010).

RESULT AND DISCUSSION

23 symptomatic samples collected from six cultivars: Cabernet, Sauvignon B**,** Nanasaheb Purple, Thopmson seedless, Sharad Seedless (SS-N) and Flame Seedless, reacted positively against GLRaV 4-9 antisera in DAS-ELISA. DAS-ELISA positive samples were subjected to two step PCR and amplified CP gene of GLRaV-4. Sequencing results showed the presence of 819 bp nucleotides (nt) in the CP gene of Indian isolates (Sharadseedless (SS-N) and Flame seedless) of GLRaV-4. CP gene of GLRaV-4 from the samples of Baramati A, Baramati E, Pune G, Pune I, Fantasy Seedless and Krishna Seedless was also amplified, cloned and sequenced. These isolates of GLRaV-4 were also of 819 bp encoding 272 amino acids (aa). Amplification could not be observed for the corresponding genes of GLRaV-5,-6, and -9. Thus the present investigation revealed that other than two previously reported viruses (GLRaV-1 & GLRaV-3) only GLRaV-4 is present in the GLD symptomatic vines of India and GLRaV- 5, -6, and -9 is conspicuous by its absence. Presence of GLRaV-2 was not tested which needs to be investigated further. The coat protein sequences of GLRaV-4 obtained from 9 Indian isolates shared maximum identity of 99% at nucleotide level and 97-98% at aa level to the LR106 isolate of USA (GenBank accession FJ467503.1). All the 9 Indian isolates shared 98-100% at nt level and 97-100% at aa level among

themselves. When CP sequences of these GLRaV-4 isolates were analyzed with the related ampeloviruses i.e. GLRaV-5,-6,-9,-Pr,-De and GLRaV- Car, Indian isolates shared 75-82% and 68-72% identities at aa and nt level, respectively. The respective sequences were found to be closely related to GLRaV-5 (82-83% amino acid similarity) and GLRaV-9 (81% amino acid similarity). The phylogenetic tree based on CP constructed using Maximum likehood (ML) (Nei et al. 2000) analysis revealed that all the ampeloviruses separated into a two distinct cluster, subgroup I and II (Fig.1). The isolates belonging to GLRaV-4,-5,-6,-9,-Pr,-De and –Car forms a tight cluster within subgroup I, which is distantly related to another cluster comprising accessions of GLRaV-1 and GLRaV-3 in subgroup II. All the 9 GLRaV-4 CPs of Indian origin grouped into the same sub cluster of subgroup I along with LR106 USA isolate. The respective sub cluster of subgroup I, shared a genetically close relationship with GLRaV-5 and GLRaV-9.

A higher degree of amino acid percent similarity in the coat proteins and restricted phloem limitation of ampeloviruses suggest possibility of genetic exchange (Martin et. al. 2009). When the 43 CP sequences of ampeloviruses were analyzed for possible recombination event, a intraspecies recombination within GLRaV-4 CPs was identified simultaneously by four algorithms of RDP4 (RDP,Maxchi, Chimaera, SiScan) with highest p value of 0.05 in which Indian isolate SS-N was a minor donor to the Italian recombinant. The recombination detected in the present study might have serious evolutionary consequences in the GLRaV-4 population.

This is the first molecular evidence of the presence of GLRaV-4 in Indian vineyards. Present information will be useful for designing the indexing programmes for grapevine in India and development of reliable detection system.

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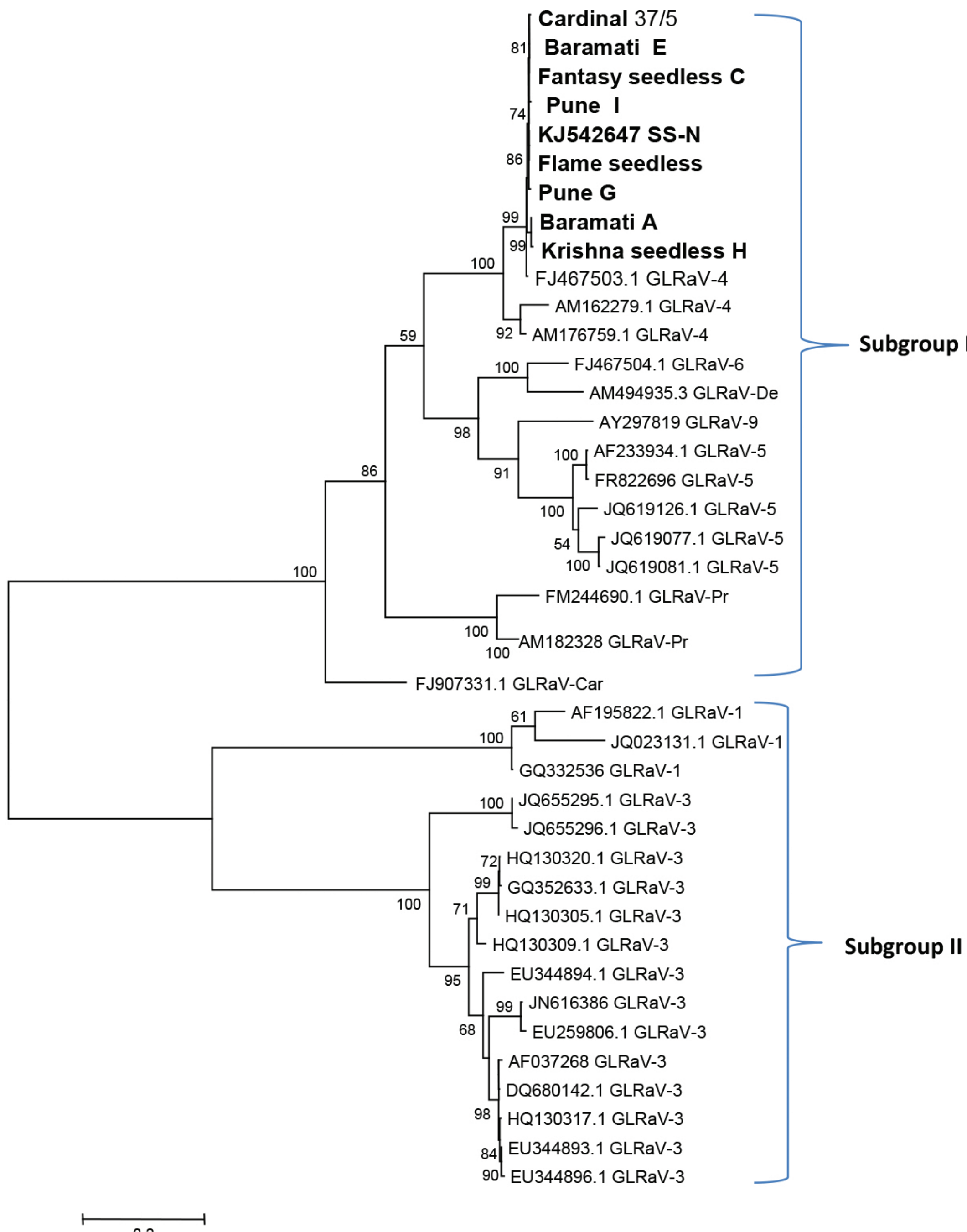

Figure. 1: Phylogenetic tree inferred with Maximum likelihood analysis based on homologous sequences corresponding to coat protein (CP) gene datasets using nucleotide sequences of ampeloviruses. Indian isolates under present study has been shown in bold letters. Only >50 % bootstrap values are shown here.

OP 20 - Genetic variability and divergence of Chilean isolates of *Grapevine rupestris stem pitting-associated virus*

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INTRODUCTION

Grapevine rupestris stem pitting-associated virus (GRSPaV) is known to be a widespread virus and has been proposed to co-evolve together with grapevine (Gambino et al., 2012). It belongs to the "rugose wood complex", a group of viruses and/or syndromes associated with alterations of trunk in vines. Additionally, due to the high genetic variability associated to this virus, different diseases have been reported depending on the lineage and the grapevine cultivar infected (Bouyahia et al*.,* 2005; Morelli et al., 2011). Several studies have derived in a four-group genetic classification (Meng et al., 2006; Nolasco et al., 2006). In Chile, GRSPaV was first detected in 2008 and since then, as expected, several detections have been done in different grapevine cultivars (Fiore et al., 2008). However, no information of genetic variability has been established associated with Chilean isolates of GRSPaV. Therefore, the objective of this research is to establish phylogenetic distribution of Chilean isolates of GRSPaV.

MATERIALS AND METHODS

One hundred and ten samples were collected from table and wine grape varieties between the regions of Atacama and Maule. Phloem scrapings from mature canes were used for virus testing. Total nucleic acid extraction was performed using silica capture method (Mackenzie et al., 1997). Specific detection of GRSPaV was performed according previously described primers (Boscia et al*.,* 2001). Molecular characterization was done using PCR primers reported by Lima et al. (2006), which partially amplified (776-bp) the helicase subunit of RdRp coding region (*Hel*). PCR fragments were purified and cloned in pGEM-T Easy kit (Promega). Five clones per isolate were sequenced to determine the eventual presence of more variants of the virus infecting the same sample. Molecular analyses were performed using MEGA6.0 tools (Tamura et al., 2013)

RESULTS AND DISCUSSION

GRSPaV was detected in 65 out of the 110 samples tested (59.1%). Nucleotide identity comparison and neighbor joining analyses carried out with the detection amplicons, gave a distribution of Chilean isolates in three groups (data not shown). Thereafter, 15 representative isolates from the three groups were randomly selected for a more exhaustive genetic analysis using *Hel* coding sequence. Figure 1 shows the phylogenetic distribution obtained in maximum parsimony analysis. Reference isolates used correspond to complete genome sequences available in GenBank and were edited to perform alignments and phylogenetic trees. In all cases, there was no sequence difference between the five cloned fragments of GRSPaV from each sample. Topologies of trees obtained with reference isolates using complete genome and *Hel* region sequences, were homologous (data not shown). Thus, *Hel* sequence oriented analysis appears to be robust enough to consider this sequence as a good indicator for phylogenetic analyses. Due to the high genetic distance observed among Syrah and PN isolates and unlike with previously reports about phylogeny classification of GRSPaV, we propose five groups: GRSPaV-1 (I), BS (II), SG1 (III), PN (IV), and Syrah (V). Chilean isolates were distributed in the groups GRSPaV-1 (most of them), SG1 and BS. Two samples, RSP-HEL 7056 and RSP HEL 6584, were divergent isolates inside GRSPaV-1 group (Figure 1). In addition, deduced aminoacid phylogenetic analyses showed a change of group of RSP HEL 6584 isolate, which shifts from GRSPaV-1 to BS and grouped with HEL RSP 6582 (data not shown). This trend must be confirmed by using, in the phylogenetic analysis, of the complete amino acid sequence of *Hel*. Finally, it is important note the common geographic and cultivar origin of the samples RSP HEL 6584 and RSP HEL 6582, the last one markedly associated with BS group. The results showed a high genetic variability among Chilean isolates and a genetic divergence for two of them. To our knowledge, this is the first molecular characterization study about GRSPaV from South America.

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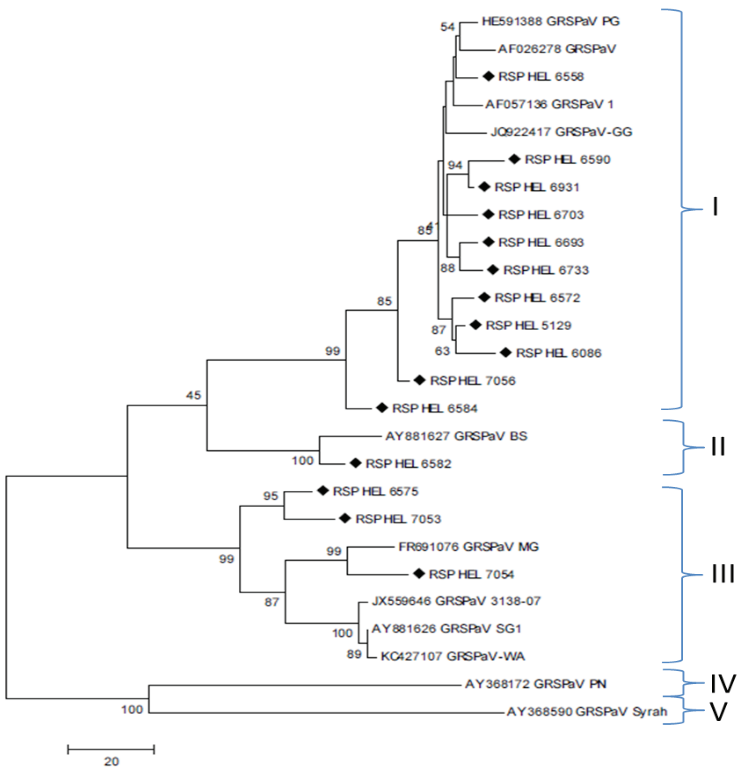

Figure 1. Maximum parsimony analysis of *Hel* sequences (776-bp) of GRSPaV isolates with 500 bootstrap replicates. Chilean isolates are marked with a filled diamond. Reference isolates information can be obtained by the corresponding accession number.

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OP 21 - Australian shiraz disease: Detection of Grapevine virus A in Shiraz vines showing a Corky Bark-like syndrome

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INTRODUCTION

'Shiraz' (syn. Syrah) is the principal wine grape variety in Australia. In 2013 it yielded 432,000 tonnes with an increase of 14% over 2012 (Anon., 2013). Unfortunately, this variety is sensitive to attack by a number of viruses including Grapevine virus A (GVA, Vitivirus, Betaflexiviridae), while many *grapevine varieties* and rootstocks infected by GVA are *symptomless.* In 2003, this laboratory reported the association of GVA with a syndrome in Shiraz which was spreading in South Australia (Habili et al., 2003; Habili and Randles, 2004). Infected plants showed delayed growth in spring, reddening of leaves, green canes and a rubbery texture in autumn; a syndrome not dissimilar to Shiraz Disease reported from South Africa (Goszczynski and Jooste, 2003). In Australia, the incidence in samples received increased from 3.4% in 2001 (728 samples tested by RT-PCR) to 16.6% in 2004 (1279 samples tested). The disease was named Australian Shiraz Disease (ASD) in 2006 (Habili, 2006). Here we report the results of testing Shiraz samples which show symptoms resembling corky bark (CB), a disease known to be associated with Grapevine virus B (GVB, Vitivirus) (Bonavia, 1996). Since the affected vines only had GVA and GRSPaV, we conclude that CB is not a reliable indicator for GVB.

MATERIALS AND METHODS

All Shiraz canes were sampled in late autumn in South Australia when ASD symptoms were expressed more strongly. At least 10 symptomatic and 10 asymptomatic vines were randomly selected for testing. The screening for viruses was done by RT-PCR (Habili and Randles, 2012) and for the GVA detection we used the specific primers designed by Minafra as described previously (Habili et al, 2003). An ELISA kit (Agri-Analysis, Davis, California) confirmed the RT-PCR results for GLRaV-3.

RESULTS AND DISCUSSION

When adjacent asymptomatic and infected 'Shiraz' vines on 'Chardonnay' rootstock were tested for 12 viruses (Habili and Randles, 2002) as well as for Australian grapevine yellows phytoplasma, grapevine rupestris stem pitting-associated virus (GRSPaV) alone was detected in the symptomless vines, while both GVA and GRSPaV were present in the diseased vines (Table 1, A & B). The diseased 'Shiraz' vines lost 98% of their yield in 2010, six years after grafting in McLaren Flat (Habili, unpublished).

In 2014, a privately owned Shiraz vineyard at the Barossa Valley which was showing typical ASD symptoms was visited. Detailed observation of the symptoms revealed the presence of swollen cracks on the stems which resembled CB (Tanne et al., 1993), a disease which is known to be associated with GVB (Bonavia et al., 1996). CB disease is under quarantine in Australia and any diseased imported material is destroyed. No GVB was detected in these samples by RT-PCR, but the tests showed that all 10 samples from infected vines were positive for GRSPaV and GVA, while all asymptomatic vines from the same vineyard had only the background virus, GRSPaV (Table 1; A & B). The unequivocal association of GVB with CB is in question as we have previously detected symptomless isolates of this virus in Australia (Shi et al., 2004).

In 2015, two different Shiraz vineyards displaying ASD were surveyed. One vineyard at McLaren Vale was infected with the following three viruses: GRSPaV, GVA and grapevine leafroll-associated virus 1 (GLRaV-1) (Table 1, C). Another vineyard in the Riverland, 250 km north east of Adelaide, was also showing ASD symptoms. Test results showed that the samples from this vineyard were also infected with three viruses, GRSPaV, GVA as well as GLRaV-3, rather than GLRaV-1 (Table 1; compare C and D). The CB-like symptoms on canes, a new descriptor for ASD was invariably associated with GVA. Symptomless canes from Vineyard D were tested positive for GLRaV-3 in 9 out of 16 samples, indicating that this leafroll virus was not linked to the CB-like syndrome. We conclude that the CB symptom is not a reliable indicator of GVB (see also Shi et al., 2004). Even though the vines showed splitting of canes (Table 1), GVB was not detected. We are applying the Tanne et al. (1993) micrografting technique using the LN33 (Couderc 1613 X Thompson Seedless) indicator to establish if the CB symptom can be expressed in the absence of GVB.

Table1. Corky bark-like symptom, a new descriptor for Australian Shiraz Disease, in vines tested positive for grapevine virus A

Sample	Virus (disease)[1]	Location	Rootstock	The CB-like symptom on canes in late autumn
A	GRSPaV (asymptomatic)	All locations	Own-roots	
B	GRSPaV + GVA (Shiraz Disease)	Barossa Valley, McLaren Flat	Chardonnay	
C	GRSPaV + GVA + GLRaV-1 (Shiraz Disease)	McLaren Vale	Own-roots	
D	GRSPaV + GVA + GLRaV-3 (Shiraz Disease)	Berri, Riverland	Ramsey	

[1] The samples were tested for 12 viruses as described by Habili and Randles (2002).

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OP 22 - Grapevine leaf mottling and deformation and *Grapevine Pinot gris virus*: an update on an emerging Mediterranean disease and a new virus

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In the last years, a group of newly detected viruses, either alien to or already present and emerging in the Mediterranean basin, were brought to the fore due to: (i) increased movement and exchange of infected propagation material; (ii) implementation of certification protocols which, by knocking out a number of regulated viruses from sanitized stocks may have favoured the expression of symptoms previously masked by those elicited by the agents of widespread diseases such as leafroll, infectious degeneration and rugose wood; (iii) the advent of new generation sequencing (NGS), a technique that was instrumental for the discovery of the novel DNA viruses *Grapevine red blotch-associated virus* (GRBaV). *Grapevine vein clearing virus* (GVCV). Grapevine Roditis leaf discoloration-associated virus (GRLDaV), and the RNA virus *Grapevine Pinot gris virus*, (GPGV).

Grapevine Pinot gris virus (GPGV) is a recently discovered Trichovirus seemingly associated with a new grapevine disease characterized by stunting, chlorotic mottling and leaf deformation (Martelli, 2014). The disease has been observed in Trentino (north-eastern Italy) since 2003 in vines of cv. Pinot gris that tested negative for the relevant grapevine clostero-, ampelo-, nepo- and vitiviruses. Genomic characterization of GPGV showed that the virus is related to *Grapevine berry inner necrosis virus* (GINV), another grapevine Trichovirus reported only from Japan and transmitted by the eriophid mite *Colomerus vitis*. GPGV genome consists of three overlapping open reading frames (ORFs) encoding proteins having, respectively, the putative functions of replication (ORF1), movement (ORF2) and coating of the viral RNA (Giampetruzzi *et al*., 2012).

GPGV discovery started a number of studies, which led to the description of the virus and the seemingly related disease, denoted Grapevine leaf mottling and defomation (Martelli, 2014), in several European and Mediterranean countries. In Italy, besides Trentino, the disease was observed in vines from Emilia Romagna, Friuli Venezia Giulia, Veneto, Lombardia and Apulia on the cvs Pinot gris, Traminer, Pinot noir, Chardonnay, Tocai, Glera and in the table grapes cvs Black Magic and Supernova (Morelli et al., 2014; Saldarelli *et al*., 2015). In Slovenia GPGV was found infecting several cvs including Pinot gris, Sauvignonasse and Muscat blanc, with an extensive spread of the disease in south western part of the country where symptoms were observed already in 2001 (Mavric Plesko et al., 2014). GPGV was reported in Slovakia and in the Czech Republic without any clear-cut association with symptoms (Glasa *et al*., 2014). In Korea, GPGV was found in the table grape cv Tamnara with symptoms of berry necrosis similar to those induced by GINV (Cho *et al*., 2013). The virus has also recently been reported from France on cvs Merlot and Carignan (Beuve et al., 2015) exhibiting fanleaf-like symptoms as well as in Turkey on cvs Pinot noir, Chardonnay, Muscat of Hamburg and two local cvs. Emir and Kadınparmağı (K. Caglayan, personal communication) and Greece (V. Maliogka, personal communication).

Since its discovery, GPGV was detected in vines simultaneously infected by *Grapevine Rupestris stem pitting-associated virus* (GRSPaV), *Grapevine Syrah virus 1* (GSyV-1), *Grapevine Rupestris vein feathering virus* (GRVFV) and the two viroids *Hop stunt viroid* (HSVd) and *Grapevine yellow speckle viroid 1* (GYSVd-1), which is consistent with a "background virome" frequently found in grapevines. Moreover, the virus was found also in some symptomless vines next to symptomatic infected vines. GPGV co-infections with viruses associated with leafroll and infectious degeneration were found in Slovakian and Czech vines and recently in France, where fanleaf-like symptoms were observed in cv. Merlot vines affected by GPGV and *Tomato black ring virus* (TBRV). Therefore, since GPGV was detected in vines simultaneously infected by other viruses it was not possible, in these cases, to associate ultimately any specific symptoms with the presence of the virus. The emerging scenario describes an inconclusively defined association of GPGV with symptoms of stunting, chlorotic mottling and leaf deformation. In a recent research the hypothesis of the existence of symptomless or symptomatic GPGV isolates was confirmed by phylogenetic and biological (indexing) studies in vine accessions from Trentino (Saldarelli *et al*., 2015). Concurrently, a further study in Friuli Venezia Giulia reported the widespread presence of GPGV and a significantly higher virus titer in symptomatic vines, which suggests the presence of different viral isolates (Bianchi *et al*., 2015).

The aim of the present review is to summarize the current knowledge on GPGV and the disease it is associated with and to update the available information with recent achievements on etiology of the disease, its spreading in the vineyards, on GPGV transmission by the eriophyid mite *Colomerus vitis* and the development of an anti-GPGV CP specific antiserum.

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OP 23 - Identification and determination of full-length sequence of three Grapevine viroids in a decade old bottled Cabernet Sauvignon Wine

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INTRODUCTION

Of the six viroids isolated from grapevine, the most prevalent are *Grapevine yellow speckle viroid 1* (GYSVd-1), an *Apscaviroid* and *Hop stunt viroid* (HSVd), a *Hostuviroid* (Little and Rezaian, 2003). A new member of apscaviroids, *Grapevine latent viroid*, has recently been described (Zhang et al., 2014). In an earlier report we described the detection of a 220 nt sequence from GYSVd-1 in various sources of wines (Habili et al., 2012). Knowing that full-length viroids are highly base-paired and hence less prone to the ribonuclease digestion, we decided to search for full viroid sequences in wines. Here, we report the presence of complete sequence of GYSVd-1, HSVd and Australian grapevine viroid (AGVd) in a Cabernet Sauvignon wine sample made in 2004.

MATERIALS AND METHODS

A wine sample of Cabernet Sauvignon was obtained from the Australian Wine Research Institute (Adelaide, South Australia). This wine was made in 2004 by a Victorian winery (Yarra Glen, Australia). Total nucleic acids were extracted from the wine sample as described before (Habili et al., 2012). For this purpose, five ml wine batches were dialysed to dryness against solid PVP 40 and resuspended in the lysis buffer of McKenzie et al (1997). Viroid amplicons obtained from single tube RT-PCR were cloned using the pGEM-T vector system (Promega) and sequenced by AGRF (Adelaide, Australia). For the detection of each viroid by RT-PCR the following primers were used (Table 1):

Table 1 Specifications of the viroid primers used in this study

Tested viroid	Primer name	Sequence(5'–3')	Amplicon (nt)	Reference
GYSVd-1	YSVdA YSVdR	CCGCCCAAAGCCCTTTTTCTTT AAGAGACCAAGTCCGCTCGAC	367 (367)[1]	This report
GYSVd-2	GYSVd-2-P1 GYSVd-2-P2	ACTTTCTTCTATCTCCGAAGC CCGAGGACCTTTTCTAGCGCTC	375 (363)	Jiang et al., 2009
AGVd	cl- hl+	GTCGACGACGAGTCGCCAGGTGAGTCTT GTCGACGAAGGGTCCTCAGCAGAGCACC	375 (369)	Rezaian et al., 1992
HSVd	HSVd-78P HSV-83M	AACCCGGGGCAACTCTTCTC AACCCGGGGCTCCTTTCTCA	300 (297)	Sano et al., 2001

[1]Actual size of each viroid genome is given in the brackets.

RESULTS AND DISCUSSION

The Blastn analysis showed that the complete sequence of GYSVd-1 detected in a Cabernet Sauvignon wine had 99.4% identity with 'Gahro' isolate of this viroid (KF916042) reported from a native table grape variety in Iran. Its predicted secondary structure and five functional domains are indicated in Figure 1.

There were two nucleotide substitutions; A234->G and a synonymous substitution of A362->U in the entire genome.

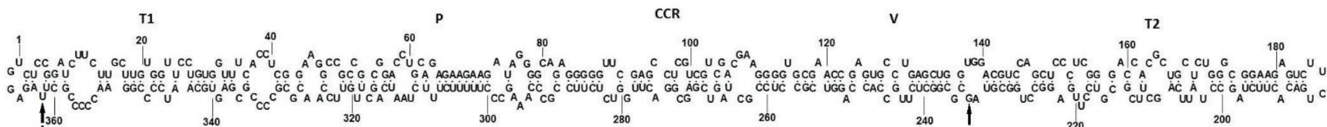

Figure1. Predicted secondary structure with lowest free energy for a GYSVd-1 isolate detected in a 2004 wine. Two nucleotide changes at positions 234 and 362 compared with those from an Iranian table grape are shown by the ascending arrows. The five structural-functional domains (T1, P, CCR, V and T2) are indicated on top.

The sequence of the full length genome of AGVd detected in the same wine sample showed 99% identity with 'Meyme' isolate from a table grape in Iran (Acc. # KF876034). These are intriguing results as they show that the sequences of each viroid isolate in the Iranian own-rooted native table grapes are highly similar to those present in a Cabernet Sauvignon blotted wine in Australia. It seems that the viroids were not under environmental pressure to induce significant diversification. The full-length amplicon of HSVd with 98% similarity to isolate H1 from grapevine in China was also obtained. GYSVd-2 was not detected using the specific primers given in Table 1. Attempts to detect apple scar skin viroid in this wine or any other wine batches that we reported previously (Habili et al., 2012) were unsuccessful.

Detection of full-length sequence of viroids with broad host range, such as HSVd (Rubio et al., 2015) in wines could indicate a potential new route for these infectious agents to enter new environments and regions.

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OP 24 - Is Grapevine red blotch-associated virus the causal agent of red blotch disease?

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INTRODUCTION

Grapevine red blotch-associated virus (GRBaV) is a recently discovered monopartite single-stranded DNA virus and a putative member of a new genus in the family *Geminiviridae* (Sudarshana et al., 2015). This virus is associated with the newly recognized red blotch disease that is present in major grape-production regions in North America (Krenz et al. 2014, Sudarshana et al., 2015) and in several grapevine accessions introduced to the United States (Al Rwahnih et al., 2015). Extensive vineyard surveys in the United States showed a close association of GRBaV with diseased vines (Al Rwahnih et al. 2013) but information on disease causality is lacking. To address this issue, we investigated the etiological role of GRBaV in red blotch disease. Here, we report our efforts to cure GRBaV-infected grapevines and to fulfill Koch's postulates by using infectious partial dimer constructs of the viral genome in agroinoculation experiments to assess the causative role of GRBaV in red blotch disease.

MATERIALS AND METHODS

For virus elimination experiments, shoots of actively growing greenhouse-grown GRBaV-infected *Vitis vinifera* cv. Cabernet franc were collected and disinfected, and microshoot tips were dissected under a stereoscope for establishment and micropropagation in tissue culture (Alzubi et al., 2012).

For agroinoculation experiments, shoots of greenhouse-grown *Vitis vinifera* cultivars Cabernet Sauvignon, Cabernet franc, Syrah, Chardonnay, and Pinot noir, and rootstock genotypes SO4 and 3309C that tested negative for GRBaV by PCR (Krenz et al., 2014) were similarly established in tissue culture and micropropagated (Alzubi et al., 2012). The same vines also tested negative for *Grapevine leafroll-associated virus 1* (GLRaV-1), GLRaV-2, GLRaV-3, GLRaV-4, GLRaV-7, *Tomato ringspot virus*, *Tobacco ringspot virus*, *Grapevine fanleaf virus* (GFLV), *Arabis mosaic virus*, and *Grapevine virus A*, as shown by double antibody sandwich (DAS)-enzyme-linked immunosorbent assay (ELISA) using specific antibodies and reverse transcription (RT)-polymerase chain reaction (PCR) with appropriate primers.

Partial dimer constructs of the genome of GRBaV isolates NY358 and NY175 were engineered and cloned into a binary plasmid for mobilization into *Agrobacterium tumefaciens* strains LBA4404 or C58. Isolates NY175 from *V. vinifera cv.* Merlot and NY358 from *V. vinifera cv.* Cabernet franc belong to GRBaV phylogenetic clades I and II, respectively (Krenz et al., 2014). Tissue culture-micropropagated grapevines (30-40 per genotype) showing 4-6 leaves were selected for agroinoculation experiments using vacuum-assisted infiltration. Alternatively, grapevine tissue was gently pricked with needles dipped in a solid agrobacterium culture grown on a Petri plate. A β-glucuronidase gene construct containing an intron was used as control to optimize conditions for agroinfiltration-mediated delivery of DNA. Constructs of both genomic RNAs of GFLV were used as negative control in agroinfiltration experiments. Following agroinfiltration and/or pricking, plants were maintained at 25±2°C and 33-45 $\mu Em^{-2}s^{-1}$ (16-h photoperiod) in a tissue culture growth room for 2-3 months prior to establishment in a greenhouse for symptom observations and testing.

The presence of GRBaV was tested by PCR in newly developed leaves of agroinoculated grapevines by using specific primers designed in the putative coat protein and replicase-associated genes, and the 16S ribosomal RNA used as a housekeeping gene (Krenz et al., 2014). Plants were tested 3-10 months post-agroinfiltration and some of them were also tested after one or two dormancy periods. The full-length genomic sequence of some of the GRBaV progeny was determined in a few selected agroinfected plants by rolling circle amplification, cloning and sequencing.

RESULTS AND DISCUSSION

A population of 54 Cabernet franc plants was obtained after virus elimination treatment by microshoot tip culture. None

of the plants derived from infected vines expressed disease symptoms (leaf reddening) in tissue culture, but some did following transfer to the greenhouse while others remained asymptomatic. After one dormancy period, 13 out of 54 vines obtained after microshoot tip culture, tested negative and 41 tested positive for GRBaV by PCR. Plants that were PCR positive exhibited typical red blotch symptoms (interveinal reddening), while PCR negative plants were asymptomatic, indicating that GRBaV can be eliminated by microshoot tip culture.

Following optimization of agroinoculation conditions, tissue culture-grown plantlets were inoculated with one of the two GRBaV bitmer constructs. A number of treated vines of Cabernet Sauvignon, Cabernet franc, Syrah and Chardonnay showed red blotch-like symptoms at 1-3 months post-treatment. Foliar symptoms consisted of interveinal reddening in red-berried cultivars and chlorotic spots in the white-berried cultivar Chardonnay. Unlike for wine grape cultivars, agroinoculated SO4 became symptomatic (chlorosis and cupping) only after one dormancy period, whereas agroinoculated 3309C remained asymptomatic.

Some of the grapevines agroinfiltrated with the NY358 construct (28-76%) tested positive for GRBaV by PCR. All the PCR-positive plants were symptomatic, while the negative plants were asymptomatic. None of the plants treated with GFP (0 of 237), GFLV-derived constructs (0 of 476) or untreated plants (0 of 56) exhibited red blotch-like symptoms, nor those that were assayed tested positive for GRBaV in PCR. The virus detected in symptomatic, agroinoculated vines was further characterized by sequencing. Sequence analysis indicated a 99.6-99.9% identify with the partial dimer construct used as inoculum in agroinfection assays, indicating that the recovered GRBaV variant is nearly-identical to the engineered inoculum. Agroinfiltration experiments with the NY175 construct are under way.

Together, our findings are consistent with our hypothesis that GRBaV is the causal agent of red blotch disease. In microshoot tip cultured plants, the absence of detected GRBaV correlated with the absence of symptoms. In agroinfiltrated plants, the detection of GRBaV correlated with symptoms and virus progeny nearly identical in sequence to the inoculated partial dimer genomic construct was obtained from agroinfiltrated plants.

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OP 25 - Sequence diversity and relationships among Grapevine red blotch virus isolates from vines within and outside a diseased vineyard

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INTRODUCTION

Grapevine red blotch virus (GRBaV) is an emerging virus of *Vitis* L. spp. in North America that is associated with delayed fruit maturation, reduced fruit juice sugars, and leafroll-like foliar symptoms (Sudarshana et al., 2015). GRBaV is a putative geminivirus (family *Geminiviridae*), with a single-stranded, circular DNA genome. The virus is graft transmissible and can be disseminated with planting material. Spread of GRBaV is suspected in certain vineyards but a vector of epidemiological significance has yet to be identified. The detection of the virus requires polymerase chain reaction (PCR)-based diagnostic methods. Among the two distinct phylogenetic clades of the virus, isolates share 91% or greater nucleotide sequence identity (Krenz et al., 2014)a sample processing strategy and multiplex polymerase chain reaction assay were developed. Forty-two of 113 vine samples collected in or received from seven of the United States were shown to harbor the virus, demonstrating the virus is widely distributed across North America. Phylogenetic analyses of a viral replication-associated protein (Rep.

Although GRBaV is widespread in the major grape growing regions of the United States, its origin remains unknown. There are no confirmed reports of the virus detected in weed hosts or other free-living plants. The virus is clearly present in commercial stocks from which planting material is derived (Stamp and Wei, 2014), but it remains to be demonstrated to what extent there are natural reservoirs of the virus and to what extent the virus is able to spread from plant to plant in the field.

We observed a vineyard with a gradient of symptomatic, GRBaV-infected vines clustered at one end, and this planting is adjacent to a riparian wetland. The objectives of this study are to determine the sequence diversity of GRBaV isolates from vines within this vineyard, and to test for the presence and sequence diversity of isolates observed in non-cultivated, free-living ('wild') plants. We hypothesize there is a major (single) population of GRBaV that is common to the wild plants and those in thecommercial vineyard.

MATERIALS AND METHODS

GRBaV was detected using PCR-based diagnostic and sequencing methods as previously described (Krenz et al., 2012, 2014)a sample processing strategy and multiplex polymerase chain reaction assay were developed. Forty-two of 113 vine samples collected in or received from seven of the United States were shown to harbor the virus, demonstrating the virus is widely distributed across North America. Phylogenetic analyses of a viral replication-associated protein (Rep. Genomic sequences were derived from at least two independent, uncloned PCR fragments or rolling circle amplification (RCA) products that were cloned prior to sequencing.

To assess the sequence diversity among GRBaV isolates, an 844 bp fragment corresponding to nucleotide (nt) positions 2987-524 on the circular genome (2987 to 3206 and 1 to 524) of isolate NY358 (GenBank JQ901105.2) was amplified and PCR products sequenced. If positions of heterogeneity were identified, independent PCR products were prepared and sequenced for confirmation.

RESULTS AND DISCUSSION

The main vineyard for this study was planted with *Vitis vinifera* cv. Cabernet franc clone 214. Surrounding plots consisted of Cabernet franc clones 327 and 623, Cabernet Sauvignon clone 4, and Merlot clone 15. Free-living (wild) plants were collected from natural habitats bordering the main vineyard site.

Our first objective was to determine if the genetic diversity in the main vineyard was consistent with the hypothesis that

it represents a single population. We prepared nucleic acid extracts from 45 vines for sequencing. A visual inspection of sequence alignments of published GRBaV genomes revealed that the most variable portion of the genome was the non-coding, intergenic region from nt positions 3045-191 on the circular genome. In order to assess the sequence diversity of GRBaV isolates, we sequenced the 587 nt region corresponding to coordinates 2991-371. Among the 45 vines, all but one isolate were most similar to clade 2 isolates (Krenz et al., 2014)a sample processing strategy and multiplex polymerase chain reaction assay were developed. Forty-two of 113 vine samples collected in or received from seven of the United States were shown to harbor the virus, demonstrating the virus is widely distributed across North America. Phylogenetic analyses of a viral replication-associated protein (Rep, with complete sequence identity among the 44 isolates. As a point of reference, among the ten published genomes of clade 2 isolates, the average heterogeneity is 0.4 to 1.0% or 2 to 6 or more divergent nucleotides for this region. This would suggest that the virus in these vines is largely a single population. The one exceptional vine harbored a clade 1 isolate; this was likely introduced from a different source. Isolates of GRBaV have been obtained from surrounding vineyards of Cabernet franc clones 327 and 623, Cabernet sauvignon clone 4, and Merlot clone 15, and their sequence relatedness is being analyzed.

Given the clustered occurrence of symptomatic vines at the main vineyard, our second objective was to determine if GRBaV could be detected in wild plants. We sampled and tested 28 non-cultivated grapevines (Vitis spp.) from the riparian habitat bordering the vineyard and proximal to the cultivated GRBaV-infected vines. Of 28 wild grape plants tested, six were shown to be positive for the detection of the diagnostic GRBaV 'rep' and 'cp' fragments (Krenz et al., 2014)a sample processing strategy and multiplex polymerase chain reaction assay were developed. Forty-two of 113 vine samples collected in or received from seven of the United States were shown to harbor the virus, demonstrating the virus is widely distributed across North America. Phylogenetic analyses of a viral replication-associated protein (Rep. All of the GRBaV isolates from the wild plants thus far sequenced fall within clade 2, as do the majority of vineyard isolates. This clearly shows that wild grapes have the potential to serve as reservoirs of GRBaV. Additional sequencing of wild plant isolates will reveal the relationship with isolates from cultivated vines and allow an assessment of whether they appear to be part of a single population.

ACKNOWLEDGMENTS

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OP 26 - Detection and characterization of Chilean isolates of grapevine viroids.

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INTRODUCTION

Grapevine is the most important fruit crop in Chile, associated with pisco, wine and table grape production. Sanitary status has been previously reviewed in relation with viral and phytoplasmic diseases (Fiore et al., 2008; Gajardo et al., 2009) but none of them considered viroidal diseases. Viroids are the smallest pathogens that can replicate autonomously in plants. They are circular single stranded RNAs, with no coding sequence and completely dependent of host plant replication machinery (Owens and Hammond, 2009). In grapevine, five viroids have been reported in different grapevine producing regions worldwide, *Grapevine yellow speckle viroid 1* and *2* (GYSVd-1, -2), *Hop stunt viroid* (HSVd), *Australian grapevine viroid* (AGVd) and *Citrus exocortis viroid* (CEVd), but in South America there is not information about viroids infecting grapevine. Thus the objective of this research was to detect and characterize the viroids present in the main grape producing areas in Chile (Koltunow y Rezaian, 1988; Koltunow and Rezaian 1989; Rezaian, 1990; Sano et al., 2001).

MATERIALS AND METHODS

One hundred and ten samples were collected mainly among the regions Atacama (with a high planted area of table grape vines) and Maule (known for its wide distribution of wine production). Total nucleic acid extraction was performed using silica capture method (Mackenzie et al., 1997). Specific detection of each viroid was performed according previously described primers (Astruc et al., 1996; Eiras et al., 2006). PCR fragments were purified and cloned in pGEM-T Easy kit (Promega). Five clones per isolate were sequenced to avoid problems in the sequences due to multiple infections. Molecular analyses were performed using MEGA6.0 tools (Tamura et al., 2013).

RESULTS AND DISCUSSION

Four of the five viroids analyzed were successfully detected. HSVd was widely detected in Chilean grapevine with no distinction about cultivar or geographic distribution. Only CEVd was not detected in all samples tested. HSVd was detected in 91.0% of the samples, followed by GYSVd-1 (20%), GYSVd-2 (10.9%), and AGVd (9.1%) (Table 1).

Table1: Viroids detection in Chilean grapevines. [a]Number of positives versus number of tested samples. Total: ratio of positives versus all analysed samples for each viroids.

Grapevine variety	Viroids[a]				
	HSVd	GYSVd-1	GYSVd-2	AGVd	CEVd
Cabernet Sauvignon	11/12	2/12	0/12	0/12	0/12
Merlot	6/6	0/6	0/6	0/6	0/6
Carménère	5/6	0/6	0/6	0/6	0/6
Pinot noir	14/14	4/14	2/14	1/14	0/14
Syrah	15/15	10/15	1/15	1/15	0/15
Sauvignon blanc	9/9	0/9	1/9	1/9	0/9
Chardonnay	4/5	0/5	0/5	0/5	0/5
Thompson Seedless	9/10	1/10	2/10	3/10	0/10
Flame Seedless	5/5	1/5	1/5	2/5	0/5
Crimson Seedless	9/10	1/10	2/10	1/10	0/10
Superior	4/5	1/5	1/5	0/5	0/5
Red Globe	4/5	0/5	2/5	1/5	0/5
Other varieties	5/8	2/8	0/8	0/8	0/8
TOTAL	100/110	22/110	12/110	10/110	0/110

HSVd was previously described in several crops, but in grapevine was usually detected with high prevalence (Sano et al., 2001). Thus, a high number of infected plants were expected. Sequence analyses gave phylogenetic association with two groups, according to classification proposed by Amari et al., (2001): P-H/Cit3 and Hop, both associated with grapevine isolates of HSVd. Another remarkable aspect is the high association of GYSVd-1 with variety Syrah, being detected in 10 of 15 samples analyzed (67%), all of them showing decline symptoms. Phylogenetic analyses of GYSVd-1 isolates gave an association of Chilean isolates to variant 1 and variant 3. Particularly, variant 3 was previously described as pathogenic variant in contrast with variant 1 described as asymptomatic (Szychowski et al., 1998). Seven of nine sequenced isolates were clustered in variant 3 but only one plant showed symptoms of vein banding. GYSVd-2 and AGVd were more prevalent in table grape varieties with 8 out of 12 detections and 7 out of 10 detections, respectively. Phylogenetic analysis of GYSVd-2 clustered all isolates in one group, closely related with Chinese and Australian isolates. Both, GYSVd-1 and GYSVd-2 phylogenetic analyses do not showed geographic origin association of different isolates, in agreement with information described by Jiang et al. (2009b). In the same way, Chilean isolates of AGVd clustered indistinctly in both reported phylogenetic groups, even when the isolates shared cultivar and geographic origin. This is not in concordance with previous reports, where it was proposed a geographic influence on the viroid variability (Jiang et al., 2009a).

The information obtained in this work represents the first report of grapevine viroids in Chile.

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OP 27 - Studies on *Grapevine Pinot gris virus* (GPGV) presence and its association with an emergent disease of grapevine

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INTRODUCTION

An emergent pathology characterized by stunting, leaf deformation, chlorotic mottling and mosaic was identified in 2003 in Northern Italy. The disease was recently suggested to be associated to a newly discovered virus, named GPGV (*Grapevine Pinot gris virus*) (Giampietruzzi et al., 2012). The virus has been later detected in a few European countries, thought the literature data are still scarce. The aetiology is still not completely clear; indeed, several reports showed that GPGV was found in most symptomatic plants, but also in a high number of asymptomatic grapevines. The aims of this work were to study: i) the possible correlation between the symptomatology and the GPGV titre in the plant; ii) the presence of the virus in vineyards of the Veneto region (Northeast Italy) and in some other European countries, in the past and nowadays.

MATERIALS AND METHODS

In 2013-2014 a total of about 300 vineyards, all cultivated with varieties known to be susceptible to the new disease, were visually surveyed in the Veneto region (Northeast Italy) for the presence of the symptomatology (Bertazzon et al., 2015). Symptomatic plants were identified and marked. Leaves were collected from at least one symptomatic and one asymptomatic grapevine per vineyard, and frozen at -80°C for molecular analyses. Moreover, a total of about 200 grapevine samples collected since 2002 from Italy and other European countries, and maintained in the CRA-VIT collection at –80°C as total RNA extracts, were used for testing the presence of GPGV.

Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen) following MacKenzie et al. (1997) and converted into cDNAs, as described in Angelini et al. (2004). Conventional and real-time PCR assays were performed with newly designed primer pairs targeting the GPGV coat protein and polymerase regions, respectively (GPGVCP-F2/R2 and GPGVPolF1/R2). Relative quantification of the virus on grapevine tissues was calculated with the comparative Cq ($2^{-\Delta\Delta Cq}$) method. Glyceraldehyde-3-phosphate dehydrogenase (GPDH) and cytochrome oxidase (COX), the two most stably-expressed genes, were used for the normalization.

RESULTS AND DISCUSSION

GPGV concentration

A subset of symptomatic vineyards planted with cv. Glera were chosen for viral titre evaluation. A total of 79 samples was analysed. Among them, 25 were collected from symptomatic grapevines, and 54 from asymptomatic ones. In these samples, together with the GPGV concentration, the titre of GRSPaV (*Grapevine Rupestris stem pitting associated virus*), an almost ubiquitous grapevine virus, was evaluated. The GRSPaV concentration was variable in the samples, and was never associated with symptomatology. On the other hand, the GPGV titre showed a statistically-significant difference between asymptomatic and symptomatic grapevines, being lower in the asymptomatic plants (Fig. 1).

GPGV concentration was evaluated also during the season 2014. Grapevine leaves were collected every month from three symptomatic and three asymptomatic grapevines belonging to cv. Glera growing close to each other in the same vineyard. Results showed that GPGV concentration was higher in the symptomatic grapevines, and that it remained higher throughout the vegetative season. Moreover, virus titre decreased during the season, both in symptomatic and asymptomatic plants, in a statistically-significant manner (Fig. 2). It should be noted that also the symptoms are widely described to decrease along the vegetative season, as it was possible to observe directly in the plants used for the experiment.

Both approaches confirmed an association between the emergent disease characterized by grapevine leaf mottling and deformation and the GPGV concentration, though the statistical variance was very high. Thus, the results suggested that an increase in the GPGV concentration led to the appearance of symptoms, while, conversely, a decrease in the virus led to recovery from the symptomatology. Joining this finding with the possible involvement of genetically distinct GPGV isolates in the manifestation of the disease that was recently reported (Saldarelli et al. 2014), it is intriguing to hypothesize that viral variants associated with symptoms could display a higher multiplication rate than variants which are not associated with the disease. Further studies are needed to prove this association definitively, and to asses the role played by environmental conditions in the manifestation of the symptoms.

Figure 1. Relative concentration of GRSPaV and GPGV in 79 asymptomatic and symptomatic grapevine samples of cv. Glera. Different letters correspond to statistically-significant differences ($p < 0.05$).

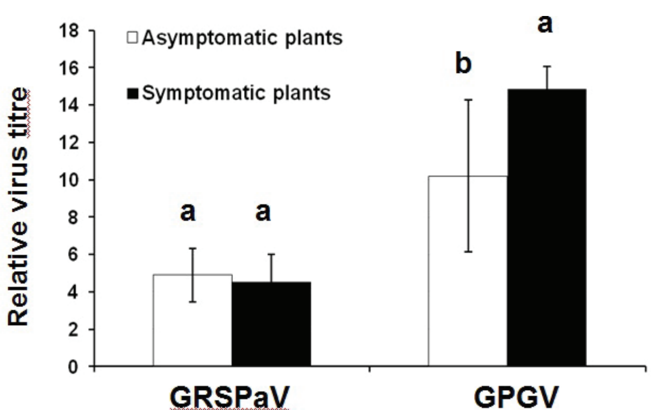
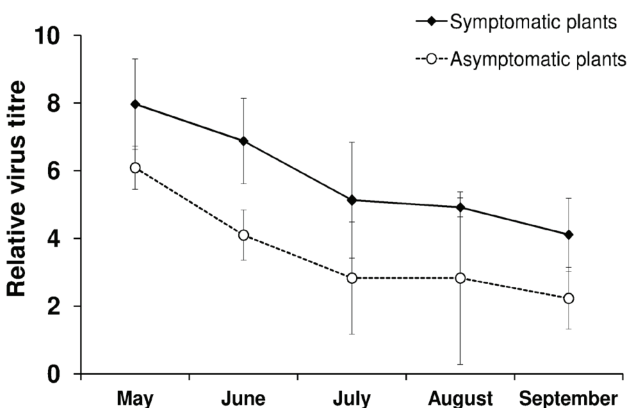


Figure 2. *Grapevine Pinot gris virus* relative concentration obtained from May to September 2014 in leaves collected from three symptomatic and three asymptomatic grapevines of cv. Glera.



GPGV presence: present and past

At first, the GPGV occurrence in the Veneto region, located in the Northeast Italy, was estimated from the analyses of 225 grapevine plants, which belonged to 28 different cultivars originating from commercial vineyards and ampelographic collections. Samples were chosen as being representative of the different cultivars and geographic grape growing areas of the Veneto region. The analyses spanned more than 10 years: indeed 150 samples collected in 2013-2014, together with other 75 samples collected in 2002-2005, were analyzed. After having ascertained the quality of RNA, analyses of the 2002-2005 samples revealed an irrelevant presence of GPGV: only one plant of cv. Cabernet franc was found to be infected. On the contrary, about 78% of the newly collected grapevines (2013-2104) tested positive. These results revealed a recent appearance of GPGV in Veneto, followed by its rapid and wide spreading in almost all the winegrowing areas of the region. A high presence of the virus in vineyards was also recently reported in Trentino (82%) and Friuli Venezia Giulia regions (Northeast Italy).

The investigation on the presence of GPGV was then extended to several European countries: 218 grapevine accessions, originally collected from local foreigner grapevine germplasm with the aim of clonal selection, were processed for the virus detection. Data obtained from samples collected before 2005 showed the presence of GPGV in Czech Republic, Macedonia, Montenegro, Spain and Ukraine, while grapevine from Croatia, France, Greece, Portugal and Serbia were negative. Analyses of grapevines collected after 2010 showed the presence of the virus in samples coming from all countries considered, *i.e.* Bosnia, Croatia, France, Greece, Portugal, Romania, Serbia, Spain and Ukraine. These results demonstrated that GPGV is occurring in much more European countries than previously though. Moreover, the data suggested that GPGV has not been present until 2005 in many viticultural areas of Europe, as it appeared to be limited to some countries of Eastern Europe. However, more detailed studies needs to confirm these preliminary data.

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OP 28 - Syrah decline in Ontario vineyards

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INTRODUCTION

Syrah decline was first discovered in the early 1990s in France (Renault-Spilmont and Boursiquot, 2002) and now has been reported in numerous countries, including the USA (Battany *et al*., 2004), Australia (Habili et al. 2006), South Africa (Goszczynski 2007), and Spain (Gramaje et al., 2009). The major symptoms of Syrah decline include swelling of the graft union, cracking and pitting of woody tissue, stem necrosis, leaf reddening, failure of the fruit to ripen fully and the death of the infected vines in 4–10 years (Battany *et al*., 2004). The etiology of this disease remains unsolved, although various viruses or viral variants and fungi have been found in Syrah decline-affected vines. *Grapevine rupestris stem pitting-associated virus* Syrah strain (GRSPaV-SY) was reported in the USA (Lima et al., 2006) and Australia (Habili et al. 2006) from vines exhibiting Syrah decline. *Grapevine Syrah virus-1* (GSyV-1), a new member of the genus *Marafivirus*, was discovered through deep sequencing of Syrah grapevines with decline symptoms (Al Rwahnih et al. 2009). *Grapevine leafroll-associated virus 3* (GLRaV-3) and *Grapevine virus A* (GVA) have been found to be associated with Shiraz disease in South Africa (Goszczynski 2007; Maree et al., 2012; Prosser et al., 2007). Fungal pathogens have also been associated with Syrah decline in Spain (Gramaje et al., 2009).

Syrah decline appeared recently in Ontario vineyards and the damage to the Syrah grapevines has become a major concern for the industry. The present study aimed to investigate the Syrah decline in Ontario vineyards. We found GRSPaV, GLRaV-3 and *Grapevine red blotch-associated virus* (GRBaV) are present in Syrah decline-affected vines.

MATERIALS AND METHODS

A preliminary disease survey was conducted in June and September 2014 in two vineyards of *Vitis vinifera* var. Syrah in Niagara, Ontario. Leaves and canes were collected for virus detection. Total RNAs were isolated from stem phloem tissues by using Spectrum™ Plant Total RNA Kit (Sigma) with our modified method (Xiao et al., in Proceedings of the 18th Congress of ICVG). The total RNAs were tested with PCR or RT-PCR for 11 viruses - GRSPaV, GRBaV, GLRaV-1, -2, -3, -4, *Grapevine fan leaf virus* (GFLV), *Grapevine virus A* (GVA), *Grapevine virus B* (GVB), *Arabis mosaic virus* (ArMV), and *Tomato ringspot virus* (TomRSV). Broad spectrum or degenerated primers were designed to target most or all the virus strains for each virus. Strain-specific primers were also used to amplify GRSPaV-SY. qPCR was done to quantify the GRSPaV, GRBaV and GLRaV-3 from Syrah grapevines.

RESULTS AND DISCUSSION

Syrah decline is present in Ontario vineyards

Two vineyards were established in 2001 to plant Syrah clone 1 and clone 100 grafted on rootstock 3309 in the major winery region of Ontario. The growers found in the past five years that the vines became less vigorous, the yield and quality of grape decreased yearly and some vines could not survive the cold winter. To diagnose the problem, we surveyed the two vineyards in June 2014 and found that 95% of vines had yellowish leaves. In September, we found that 95% of vines had leaves with red blotch, and the canopy of 25-30% of vines became uniformly red (Figure 1). The vines with red canopy had red leaves with red veins, and some of the canes showed necrosis. Almost half of the vines with red canopy did not survive the very cold winter this year.

Figure 1. Symptoms of Syrah decline. Arrows indicate the vines with red canopy.

Three viruses are detected in vines with Syrah decline

Total RNAs were isolated from cambium scraping of 10 vines each from clone 1 and clone 100 with or without red canopy (Table 1). The RNAs were tested with PCR or RT-PCR for 11 viruses infecting grapevines. GRSPaV and GLRaV-3 were found in all 20 samples tested. GRBaV was found in 16 of the 20 samples by PCR, and three of the negative samples tested positive in qPCR detection with a high Cq value, indicating very low viral titers. None of the remaining eight viruses was detected in any of the 20 samples. We then used qPCR to quantify the virus titer of GRSPaV, GRBaV and GLRaV-3 in these samples and ubiquitin was included as an internal control. No correlation, however, was found between virus titer of each of the three viruses and Syrah decline either (Table 1).

GRSPaV–SY was found to be associated with Syrah decline in several reports (Lima et al., 2006; Habili et al. 2006). RT-PCR with strain-specific primers was used to detect Syrah strain in these samples. We found that all 20 samples, regardless of having red canopy or not, had the Syrah strain (Table 1). Although the virus titers among the samples were quite different based on the intensity of the amplification products on the gel, no correlation was found between virus titer and the presence of red canopy. In a separate cloning experiment using broad spectrum primers we have identified seven GRSPaV variants including a new variant, ON1, from the two Syrah vineyards (Table 1). Further work is being done to compare the GRSPaV variants from vines with and without red canopy.

To identify the strains of GLRaV-3 in vines with or without red canopy, we cloned and sequenced the coat protein gene and found that these viral isolates were highly similar to 623 or WA-MR (Table 1). More sequencing from additional samples is needed to see if a specific GLRaV-3 strain is consistently associated with Syrah decline.

Based on the data from this study, we conclude that at least three viruses are associated with Syrah decline in Ontario vineyards. We are in the process of conducting further analysis toward the etiology of the disease through deep sequencing. Meanwhile, we are studying the temporal and spatial kinetics of the disease over years. Comprehensive analysis of all the information generated may ultimately offer a clue to the cause of Syrah decline in Ontario vineyards.

Table 1. RT-PCR and RT-qPCR detection of viruses from Syrah vines with and without Syrah decline symptoms

Sample name	Symptoms	GRSPaV			GLRaV3		GRBaV	Ubiquitin
		RT-PCR / RT-qPCR[a]	Syrah strain[b]	Other strains[c]	RT-PCR / RT-qPCR[a]	Strains[c]	PCR / qPCR[a]	RT-qPCR[ba]
Clone 1-R9-V2	red blotch, red canopy	+ / NT	+	ND	+ / NT	623	- / NT	NT
Clone 1-R9-V4	red blotch, red canopy	+ / 28.0	+	GG, ON1	+ / 26.9	ND	- / 36.7	20.7
Clone 1-R9-V5	red blotch, red canopy	+ / 28.6	+	ND	+ / 28.4	ND	+ / 33.9	21.0
Clone 1-R9-V6	red blotch, red canopy	+ / 27.4	+	MG, 47-4, OE8	+ / 28.5	ND	- / 35.8	21.0
Clone 1-R9-V7	red blotch, red canopy	+ / 26.3	+	ON1	+ / 28.5	ND	+ / 29.3	21.0
Clone 1-R9-V8	red blotch, red canopy	+ / 25.0	+	ND	+ / 27.6	ND	+ / 20.8	20.5
Clone 1-R9-V9	red blotch, red canopy	+ / 28.3	+	ND	+ / 29.2	ND	- / 33.1	20.9
Clone 1-R9-V10	red blotch	+/ 33.7	+	ND	+ / NT	623	+ / ND	NT
Clone 1-R9-V11	red blotch	+ / NT	+	ND	+ / NT	623	+ / ND	NT
Clone 1-R9-V15	red blotch	+ / 28.2	+	ND	+ / 27.7	ND	+ / 14.0	21.0
Clone 100-R17-V3	red blotch, red canopy	+ / 25.4	+	BS, GR4	+ / 29.1	623, WA-MR	+ / 15.0	24.7
Clone 100-R17-V4	red blotch	+ / 25.2	+	MG, GR4, ON1	+ / 32.0	ND	+ / 13.5	25.0
Clone 100-R17-V7	red blotch	+ / 27.6	+	ND	+ / 31.7	ND	+ / 14.1	25.0
Clone 100-R17-V9	red blotch	+ / NT	+	ND	+ / NT	ND	+ / NT	NT
Clone 100-R17-V10	red blotch, red canopy	+ / 26.3	+	ND	+ / 31.2	ND	+ / 14.2	25.1
Clone 100-R17-V11	red blotch	+ / 27.9	+	MG, 47-4	+ / 27.4	ND	+ / 13.5	25.0
Clone 100-R17-V12	red blotch	+ / 29.1	+	ND	+ / 30.0	ND	+ / 12.6	25.4
Clone 100-R17-V13	red blotch	+ / 27.7	+	ND	+ / 31.2	ND	+ / 13.1	25.2
Clone 100-R17-V14	red blotch	+ / 28.5	+	ND	+ / 32.0	ND	+ / 13.9	26.1
Clone 100-R17-V15	red blotch, red canopy	+ / 26.5	+	ND	+ / 31.2	ND	+ / 14.9	25.0

+ and – : positive and negative respectively in RT-PCR; a: the quantification cycle (C_q) values is showed; b : Syrah strain is detected by RT-PCR; c: determined by sequencing; NT: not tested; ND: not determined; Primers used are RSP35 / RSP36 and SY1659F / SY2127R for GRSPaV, GRBaV1097F / GRBaV1331R for GRBaV, LR3CP107F / LR3-CP407R for GLRaV-3, and UBI-F / UBI-R for ubiquitin.

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OP 29 - The situation of the new emerging grapevine viruses in Turkey

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INTRODUCTION

Grapevines have been cultivated in Europe and Asia for thousands of years. Turkey, due to its geographical position, is located in the center of where grape was first cultivated and processed for wine production (Köprülü, 2014). There are 25 recognized virus and virus like diseases of grapevines. Many have a detrimental effect on the vines, that is stunting, reduced vigour, malformation of the leaves and twigs, foliar discolorations ranging from reddening to chlorotic or bright yellow mottling, ringspots and line patterns (Martelli, 2014). Beside these well-described viruses, next generation sequencing has been shown to be a good option for investigating diseases of unknown etiology in grapevine (Chiumenti et al. 2012). Recently, the following novel viruses were discovered infecting grapevine by this technique and the provisional names were given as *Grapevine syrah virus-1* (GSyV-1), *Grapevine red blotch-associated virus* (GRBaV), *Grapevine vein clearing virus* (GVCV), *Grapevine pinot gris virus* (GPGV) and *Grapevine roditis leaf discoloration associated virus* (GRLDaV) (Giampetruzzi et al. 2012; Martelli, 2014; Maliogka et al. 2015). Some of these new viruses are associated to emerging diseases, whose origin, etiology, epidemiology and economic impact are still very poorly known. The main objective of this study was to evaluate the presence of these new emerging viruses in both germplasm collection and/or commercial vineyards in Tekirdağ and Hatay provinces of Turkey.

MATERIALS AND METHODS

In October 2014, 120 grapevine samples which were showing leaf deformation, chlorotic mottling on the leaves, reduced yield and quality were collected mainly from cvs. Pinot noir, Chardonay, Muscat of Hamburg, Zinfandel, Emir and Kadın Parmağı in Tekirdağ and cvs. Antep Karası and Pafu from Hatay provinces. In order to verify the presence of new emerging viruses of grapevine, the samples were tested for *Grapevine syrah virus-1* (GSyV-1), *Grapevine red blotch-associated virus* (GRBaV), *Grapevine vein clearing virus* (GVCV), *Grapevine pinot gris virus* (GPGV) and *Grapevine roditis leaf discoloration associated virus* (GRLDaV) by PCR analysis. Total RNAs were extracted from cortical scrapings of one year old shoots during dormancy using RNeasy Plant Mini Kit (QIAGEN, Valencia, CA). The synthesis of complementary DNA (cDNA) was performed by using commercial cDNA synthesis kit (ThermoSci., USA). Already published specific detection primers were used for these viruses (Engel et al., 2010, Zhang et al., 2011, Rwahnih et al., 2013, Glasa et al., 2014, Maliogka et al., 2015).

RESULTS AND DISCUSSION

In this study, among the tested samples only GPGV was detected in different grapevine cultivars collected from both collection and commercial vineyards in Tekirdağ province. Twenty two samples out of sixty were found infected only by GPGV in this province whereas not any virus was detected in Hatay. RT-PCR results showed that DNA fragments of 411 bp, 302 bp and 618 bp corresponding to the part of the coat protein (CP) gene, part of the movement protein gene (MP) and 5' UTR and the N-terminus of the replicase gene of GPGV were successfully amplified, respectively. All PCR products of GPGV were directly sequenced on both strands. The sequences were deposited in the NCBI database. All the nucleotide sequences of CP, MP and 5' UTR and N-terminus of replicase genes shared the highest sequence identity with different GPGV isolates deposited in GenBank. These results clearly showed that some foreign and local cultivars from Turkey can be affected by GPGV. It was first time detected in Northern Italy (Giampetruzzi et al., 2012) and later in Southern Italy (Morelli et al., 2014), South Korea, Slovenia, Greece (Martelli, 2014), Slovakia, Czech Republic (Glasa et al., 2013) and very recently in France (Beuve et al., 2015) and Turkey (Gazel et al., 2015, in press).

The prevalence, geographical distribution, pathogenicity, epidemiology and economic impact of GPGV are still very poorly known. The distribution of GPGV in both local and foreign grapevine cultivars in Tekirdağ province pointed out the possible transmission of this virus by arthropod vectors. The studies on the epidemiology of GPGV and the effect of GPGV on yield and quality of infected grapevines are still in progress in Turkey.

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OP 30 - *Grapevine Pinot gris virus* (GPGV) detection in Slovenia

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INTRODUCTION

Grapevine Pinot gris virus (GPGV) was first described in 2012 in Italy in association with a new disease of grapevine of an unknown etiology (Giampetruzzi et al., 2012). Since then the virus was found in several European countries in symptomatic and non-symptomatic plants including Slovenia, Slovakia, Czech Republic, France and Greece (Mavric Plesko et al., 2014; Glasa et al., 2014; Martelli, 2014; Beuce et al., 2015) The only report of GPGV outside Europe is from Korea in 2013 (Cho et al., 2013). Due to a severe outbreak of the disease in 2014 in south-western part of Slovenia we conducted a detailed screening of the presence of GPGV in this part of the country.

MATERIALS AND METHODS

Samples of symptomatic and symptomless plants of several grapevine cultivars were collected in vineyards in south-western part of Slovenia in 2012 and 2014. Samples collected in 2012, cv. Muscat blanc, Pinot gris and Pinot noir, were used for the first confirmation of GPGV infection in Slovenia. PCR products were cloned into pGEM-T vector, sequenced and sequences were deposited into the EBI database under Acc. No. HG738850 – HG738852 (Mavric Plesko et al., 2014). In 2014 visual inspection of production vineyards were conducted. Samples from selected vines were tested by RT-PCR using primers GPgV5619 and GPgV6668 (Giampetruzzi et al., 2012).

RESULTS AND DISCUSSION

Out of 42 mostly symptomatic samples of cvs. Muscat blanc, Pinot gris and Pinot noir collected in 2012, part of movement protein gene was successfully amplified from 40 samples. Amplification products of 3 samples (one for each cultivar) were cloned and sequenced. Obtained sequences showed 97,4 – 97,6% identity of nucleotide sequence and 97,1 – 98,2% identity of deduced protein sequence (Mavric Plesko et al., 2014).

Due to a severe outbreak of the disease symptoms in 2014 the monitoring of GPGV was initiated. Affected plants showed shortened internodes, poor leaf development, mottling and deformation of **References** leaves. Severely affected plants also showed poor growth. Out of 118 analysed symptomatic and symptomless samples 99 tested positive for GPGV. GPGV infection was confirmed in cvs. Rebula, Sauvignon vert, Malvazija, Pinot gris, Pokalca, Pinot blanc, Merlot, Refošk and Vitovska grganja. The monitoring was continued in 2015 and further research about the disease and virus will be conducted in the future.

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OP 31 - Grapevine vein banding in North-West Iran

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INTRODUCTION

Grapevine yellow speckle viroid-1 (GYSVd-1) and *Grapevine yellow speckle viroid-2* (GYSVd-2) are the causal agents of yellow speckle (YS) disease, which is characterized by minute yellowish spots or flecks scattered on the blade or gathering along the veins of the leaves. Symptoms of YS, first reported from Australia (Taylor and Woodham, 1972), are ephemeral and usually appear in the height of summer (Stellmach and Goheen, 1988). The concomitant infection with GYSVd-1 and/or GYSVd-2 and *Grapevine fanleaf virus* (GFLV) elicits vein banding (VB) (Krake and Woodham, 1983; Szychowski *et al*., 1995), whose symptoms consist of chrome yellow flecks localized along the main veins and progressing into the interveinal areas of the affected vines (Goheen and Hewitt, 1962). Once it was thought that such symptoms are solely incited by GFLV, but it was in 1995 that further investigation showed that viroids are also involved in the disease (Szychowski *et al*., 1995).

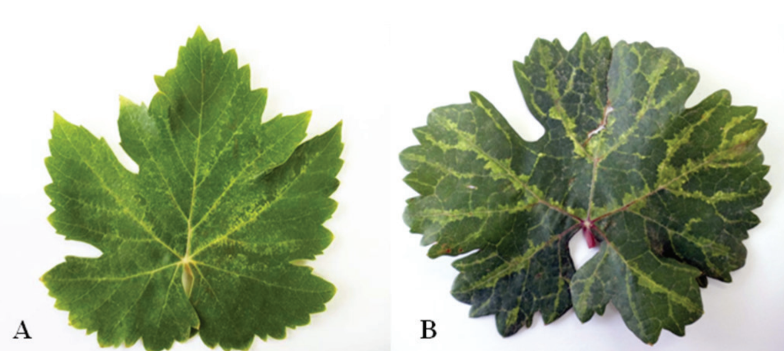
MATERIALES AND METHODS

In summer 2011, 137 samples were collected from vines showing YS and VB, as well from vines without such symptoms in West-Azerbaijan, East-Azerbaijan and Ardabil provinces (North-West Iran). Total nucleic acids (TNA) were extracted according to a protocol by Foissac *et al*. (2000) with minor modifications as described by Hajizadeh *et al*. (2012). Reverse transcription (RT) was done with random hexamer primers and polymerase chain reaction (PCR) with the specific primer pair GYSVd-1 and GYSVd-2 (Table 1) as described in Wan Chow Wah and Symons (1997) using *Pfu* DNA polymerase. The same RNA preparations from the 137 grapevines which were tested for viroid infection were further assayed for GFLV by RT-PCR using the specific primer pair (Table 1) designed by MacKenzie *et al*. (1997).

Table 1. Primers used to synthesize full-length clones of *Grapevine yellow speckle viroid* 1 and 2 (GYSVd-1, GYSVd-2) and *Grapevine fanleaf virus* (GFLV).

Viroids/virus	Primer	Sequences (5′→3′)	Position	Size (bp)	References
GYSVd-1	GV2P	TAAGAGGTCTCCGGATCTTCTTGC	356-12	366-368	Polivka et al., 1996
	GV1M	GCGGGGGTTCCGGGGATTGC	336-355		
GYSVd-2	GYSVd-2-for	ACTTTCTTCTATCTCCGAAGCCG	184-206	363	Gambino et al., 2014
	GYSVd-2-P2	CCGAGGACCTTTTCTAGCGCTC	162-183		
GFLV	C3310	GATGGTAACGCTCCCGCTGCTGCTCTT	3286-3310	312	MacKenzie et al., 1997
	H2999	TCGGGTGAGACTGCGCAACTTCCTA	2999-3024		

RESULTS AND DISCUSSION



RT-PCR showed that GYSVd-1 and GYSVd-2 occurred in 125 (91%) and 87 (64%) of the samples, respectively. GFLV was detected in 37% of the samples, confirming the previously reported widespread occurrence of this virus in north-west Iran (Sokhandan-Bashir *et al*., 2007). VB (Fig. 1B) was observed in 22 vines all of which were also infected by GYSVd-1, GYSVd-2 and GFLV, thus confirming previous reports on concurrence of these different agents in the etiology of this syndrome (Szychowski *et al*., 1995). YS symptoms (Fig. 1A), which occurred in about 10% of the tested plants, were shown to be always associated with vines infected by GYSVd-1 and/or GYSVd-2. These findings are in line with the notion that assigns to GYSVd-1 and GYSVd-2 a role in the induction of YS, and to both viroids and GFLV the genesis of VB.

Figure 1. A, representative leaf of *Vitis Vinifera* infected by GYSVd-1 and GYSVd-2 and showing yellow speckle symptoms. **B**, representative leaf of *Vitis Vinifera* infected by GYSVd-1, GYSVd-2 and GFLV showing vein banding symptoms.

Besides highlighting the large prevalence of viroids in Iranian grapevines, this study confirms the role of GYSVd-1 and GYSVd-2 in the genesis of YS. In addition, our data show that in Iran, and in other areas in which GFLV is also present, additional damage can be expected in vines simultaneously infected by this virus and by GYSVd-1 and/or GYSVd-2, essentially due to the elicitation of VB. The results reported here have been recently confirmed by multiplex RT-PCR assay (Hajizadeh *et al*., 2012), which additionally identified AGVd and HSVd in several isolates. Representative populations of these viroids have been molecularly characterized and will be shown elsewhere (Hajizadeh *et al*., 2015).

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OP 32 - Next-Generation Sequencing poised to replace biological indexing as the gold standard for virus detection in Grapevine

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INTRODUCTION

Biological indexing has historically been the Gold Standard method of choice for the detection of viral diseases ((Rowhani et al, 2005, Martin, 2012) in grapevine registration and certification programs. That bioassay identifies infection by pathological viruses by the generic disease symptoms they induce after graft inoculation of test material to a panel of indicator vines We compared that bioassay against next-generation sequencing (NGS) analysis for the assessment of viral infection. With NGS analysis, the totality of all the genomic DNA and RNA sequences in a sample is produced as a dataset by computer analysis of deep sequencing data. NGS analysis identifies viruses to the species level. The comparison between the two techniques showed that NGS analysis was superior for certification analysis by every measure tested.

MATERIALS AND METHODS

We compared the biological assay and NGS analysis in parallel, using the same15 infected grapevine accessions (shown in Table 1) for both. The bioassay employed a panel of index hosts varieties including Cabernet franc, LN 33, St George and Kober 5BB. Three to six replicates of these four indicator plants were bud chip inoculated from each of the 15 test plant. Two bud chips were grafted to each indicator plant, and the grafted plants were maintained in the greenhouse for one month to allow the graft to heal. After two to four weeks of acclimatization in a shade house, the number of surviving buds were counted; one viable bud out of two was scored as successful bud take. Successfully grafted plants were then planted in the field. Symptoms were evaluated 1 to 1.5 years after inoculation, by at least two observers. Tests in which at least two plants were symptomatic were scored as positive; if just one plant was symptomatic, the test was repeated. Six replicates of each graft inoculation from source plants carrying Grapevine leafroll-associated virus -2 and -3, GVA and GVB, Grapevine fanleaf virus, Tomato ringspot virus, Grapevine fleck virus, and GRSPaV were run as positive controls; uninfected controls were maintained in parallel and similarly scored. Herbaceous hosts were also assayed in parallel, to screen for sap-transmissible viruses.

NGS analysis involved the generation of the totality of all the genomic DNA and RNA sequences in a sample, produced as a data set by computer analysis of deep sequencing data. Pathogens were identified by comparisons of the information in the data set against the database of all known pathogen genomic sequences. The sequence dataset was derived from nucleic acid fractions extracted from bark scrapings for each of the 15 test plants. Complementary DNA was prepared using the SuperScript II Reverse Transcriptase kit primed with random hexamers and amplified using the GenomePlex complete whole genome amplification kit. Sequence data was generated by Eureka Genomics Inc. Approximately six million unique reads were generated from each sample. Reads were trimmed using the CLC Bio Genomic Workstation trimming tool, then they were mapped against the grapevine genome to subtract the host background. Contigs were then built from the unmapped reads. NCBI's BLASTx program was used to identify the viral species present. This was done by comparison of the contig sequences to reference sequences in the GenBank database. The entire process of NGS census of the viromes of each plant took less than one month to run.

RESULTS AND DISCUSSION

The biological assay identified seven viral diseases of known agronomic significance in grapevine by the disease symptoms they induced in indicator host plants (Table 1). The NGS assay identified twenty nine species and strains of viruses, viroids, and viral satellites, including a novel, previously undescribed grapevine reovirus in the same 15 test plants. It identified the leafroll viruses to the species level. It identified infections by GRBaV, GSyV-1, GRVFV and by the undescribed reovirus, all of which are beyond the scope of the biological assay. The NGS assay proved to be more accurate than the biological assay, identifying infections that were missed by the bioassay in 8.3% (5 per 60) of the grapevine indicator host tests. The bioassay failed to produce disease symptoms from the GVA infections present in the Sangiovese and the Aldo accessions, from the GRSPaV infection in the Helena accession, from the GFLV in the Semillon accession, and from the GFkV infection in the Cabernet Sauvignon (sample 3). The herbaceous bioassay detected the GFLV infection in the St George accession (viz. NGS detection of that GFLV with 55% viral genomic coverage), but it failed to detect the GFLV infection in the Semillon accession (detected with 15% genomic coverage in the NGS assay).

In aspects of the comparison between these two methods other than detection sensitivity, such as specificity and reliability, NGS appeared superior to the bioassay (Al Rwahnih et al, 2015). But the most significant distinction between the two methods was in the time to completion for each process. The biological indexing assay required nearly two years to complete under California conditions (and would require even longer in cooler climates; Golino, D., *unpublished observations*). NGS laboratory assays were completed in weeks. Due to this savings of time and to the costs involved with the grafting and maintenance of test vines in the greenhouse and then in the field, NGS analysis would likely be preferred over biological index testing for most applications.

	Accession	Variety	Infections Identified	CF	LN33	SG	K5BB	Herb.
1	LR102	Marsanne	LR GVA GVB GRSPaV	+	+	+	+	-
2	CB100	Semillion	LR GVB GFLV GRSPaV	+	+	+	-	-
3	LV89.2	Cab Sauvignon	LR GVB GFkV GRSPaV	+	+	+	nt	-
4	LR106	Thompson	LR GRSPaV	+	-	+	-	-
5	LV91.1	Cabernet Franc	LR GVA GVB GRSPaV	+	+	+	+	-
6	LV94.4	Chardonnay	LR GVA GVB GFkV . GRSPaV	+	+	+	+	-
7	CB120	St George	GVA GVB GFLV	-	+	+	+	+
8	ToSV100	Carignane	LR GRSPaV ToRSV	+	-	+	-	+
9	LR127	Sangiovese	LR GRSPaV	+	-	+	-	-
10	K5BB	Kober 5 BB	-	-	-	-	-	-
11	LV89.15	Cab Sauvignon	LR GVB GFkV GRSPaV	+	+	+	-	-
12	LR118	Helena	LR GRSPaV	+	-	+	-	-
13	LR119	Cab Sauvignon	LR GRSPaV	+	-	+	-	-
14	CB101	Aldo	LR GVB GRSPaV	+	+	+	nt	-
15	LR110	Italia 3	LR GRSPaV	+	-	+	-	-

Table 1. Biological Assay Data. Viral infections in 15 accessions scored from bioassay on Cabernet franc (CF), LN33, St. George (SG) and Kober 5BB (K5BB) indicator varieties, and on herbaceous indicator hosts. Disease symptoms on St. George occurred either on the stem or on the foliage. Infection by individual viruses was verified by both PCR tests and by the NGS procedure.

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OP 33 - Sampling guidelines and seasonal detection of Grapevine red blotch associated virus

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INTRODUCTION

Grapevine red blotch associated virus (GRBaV) is a newly described virus associated with red blotch disease. Due to foliar symptom similarity, the presence of GRBaV infection is constantly confused with leafroll disease and/or other disorders that cause reddening in red fruited grape varieties. In spite of its name, GRBaV is frequently detected in white fruited varieties. Besides the typical foliar symptoms, GRBaV has been reported to affect sugar accumulation in grapevines reducing the brix values and delaying the harvest of fruit. To determine if the detection of GRBaV is seasonal we tested GRBaV-infected vines from different sections of the vine using real time and end point polymerase chain reaction (PCR). Before this study information on the distribution of GRBaV in grapevines as well as sampling guidelines was lacking. The purpose of the study was to optimize different PCR methods and to determine the best sampling strategy for optimal detection.

MATERIALS AND METHODS

In this study the end point and quantitative polymerase chain reaction methodologies were compared. Samples were collected throughout the season from infected vines and healthy grapevine controls. During the spring and summer season the tests were performed with actively growing (green) tissue and compared with lignified canes collected in the fall and winter seasons. The following tissue samples were analyzed: leaf blades and petioles (basal and apical location); dormant and active buds, inflorescence rachis and flowers, green and lignified canes, mature cordon, and trunk. In addition, we tested newly grafted vines in which the scion was known to be infected and the rootstock had shown no evidence of infection of GRBaV.

RESULTS AND DISCUSSION

The testing results revealed the presence of GRBaV in every tissue type sample tested when collected from infected vines. In other words, the virus was detected in high concentration regardless of the tissue tested: apical shoots, apical and basal leaves; petioles from basal and apical leaves; leaf blades or veins; lignified and green canes; flowers, fruits, and/ or inflorescence rachis, etc. This data suggests that GRBaV distribution is uniform in infected grapevine tissues. Furthermore, the analyses of newly grafted vines in which the scion was known to be infected yielded equivalent PCR detection signals in the scion and rootstock portions sampled. This data suggests that the virus can move from the infected scion into the rootstock in a short period of time (less than three months). Symptoms of different grapevine varieties and sampling guidelines developed in our lab for the detection of GRBaV will be discussed.

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OP 34 - Assessment of a novel approach for viral metagenomics studies; performance on grapevine viruses

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INTRODUCTION

New sequencing technologies enable rapid viral diagnostics of a diseased plant. However, despite a continuing decrease in sequencing costs, it is difficult to justify their use in large-scale surveys without a virus enrichment technique. Since the majority of the viruses are encoded by RNA, a popular approach is to extract the double-stranded-RNA (dsRNA) replicative form to enrich the virus genetic material over the plant background (Al Rwahnih et al., 2009; Al Rwahnih et al., 2012; Coetzee et al., 2010). The traditional dsRNA extraction is time-consuming and labour-intensive. We present the preliminary results of an alternative method to enrich dsRNA from a crude sap preparation using anti-dsRNA monoclonal antibodies in a pull-down assay. The extracted dsRNA can be amplified by RT-PCR and sequenced by Next Generation Sequencing.

MATERIALS AND METHODS

A pilot study was performed on both soft-tissue plants and grapevines. Three soft-tissue plant species were selected, potato (*Solanum tuberosum*), a New Zealand native lily, rengarenga (*Arthropodium cirratum*) and dock (*Rumex* sp.), to represent a cultivated crop, an ornamental plant and a weed, respectively. The limited grapevine study was performed on six samples of grapevines from commercial vineyards (*Vitis vinifera* Chardonnay Mendoza and Malbec), and from an old germplasm collection (*V. vinifera* Pinot noir, Sauvignon blanc, Cabernet sauvignon and *V. labrusca* Fredonia).

Extraction was performed on 100 mg of freeze-dried tissue and ground to a fine powder. Monoclonal antibodies anti-dsRNA (2G4) were coated on Protein L magnetic beads for one hour at 37°C. The beads were washed (on a magnetic holder) and added to the plant extract (on Tris-buffered saline with Tween®) and incubated for one hour at 37°C. After a second wash, the protocol followed the one described by Roossinck et al (2010): the reverse transcription was performed on the beads using random primers. Samples were then treated with RNAse A. Then a PCR was then performed with a tagged primer. Samples were sequenced with HiSeq 100 bp PE.

RESULTS

Two viruses were detected in the potato sample (*Potato virus S* and *Potato virus Y*). The native lily was found to be infected with a common *Potyvirus* (*Turnip mosaic virus*) and finally the dock was infected with *Cherry leafroll virus* and a novel Macluravirus (Table 1).

From the grapevines we detected six common grapevine viruses: *Grapevine leafroll-associated virus 1*, 2 and *3* (GLRaV-1, GLRaV-2, and GLRaV-3), *Grapevine virus A* (GVA), *Grapevine rupestris vein feathering virus* (GRVFV), and *Grapevine rupestris stem pitting-associated virus* (GRSPaV).

DISCUSSION

We present here a novel approach for virus enrichment prior to NGS. The preliminary results suggest that this tool has significant potential for large-scale viral metagenomics analysis. The pilot study on the soft tissue showed a high ratio of virus reads sequenced. The tool enabled the discovery of a new host record for TuMV (*Arthropodium cirratum)* and a novel virus from the dock sample. The Macluravirus's closest relative is *Chinese yam necrotic mosaic virus* (CYNMC) with 52% amino acid identity over the coding region.

Despite a significantly lower ratio of virus to other reads from the grapevines, reflected by a lowered sensitivity for a comparable depth of sequencing, the method was successful at detecting six viruses. The results are remarkable considering that the dsRNA enrichment was performed in 2 hours from only 100 mg of dried leaf tissue.

The method is being modified to improve its sensitivity for the detection of grapevine viruses in order to be used in a large-scale survey. We believe that this new assay, with modification, represents a significant opportunity to upscale plant virus ecology studies including in woody tissues with low titre viruses.

Table 1: Viruses detected per host. Virus acronyms used: PVY: *Potato virus Y*; PVS: *Potato virus S*; TuMV: *Turnip mosaic virus*; CLRV: *Cherry leafroll virus*; GRSPaV: *Grapevine rupestris stem pitting- associated virus*; GLRaV(1, 2 or 3): *Grapevine leaf roll-associated virus* (1, 2 or 3); GVA: *Grapevine virus A*; GRVFV: *Grapevine rupestris vein feathering virus.*

Host	Virus detected
Solanum tuberosum	PVY; PVS^{A} and PVS^{O}
Arthropodium cirratum	TuMV
Rumex sp.	CLRV; Rumex virus Y (Macluravirus)
Vitis vinifera Chardonnay Mendoza	GRSPaV; GLRaV-1; GVA
Vitis labrusca Fredonia	GLRaV-2
Vitis vinifera Pinot noir	GRSPaV; GLRaV-3
Vitis vinifera Sauvignon blanc	GRSPaV; GLRaV-3; GRVFV
Vitis vinifera Malbec	GRVFV
Vitis vinifera Cabernet sauvignon	GRSPaV; GLRaV-3

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OP 35 - Failure to detect Grapevine Rupestris Stem Pitting-associated virus in Iran may give a clue to the origin of this virus

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INTRODUCTION

Grapevine rupestris stem pitting-associated virus (GRSPaV, genus *Foveavirus*, family *Betaflexiviridae*) is the most commonly occurring virus in the grapevine worldwide. Over 90% of the grapevines in Australia are infected with this virus. Most infected plants do not show symptoms, however, a few strains are associated with the stem pitting disease. The mode of transmission of this virus in vineyards is not known, but its natural spread has been observed (see Habili et al., these proceedings). The most common route of its spread is by humans via disseminating infected cuttings. Waite Diagnostics has been testing for GRSPaV in samples from Australia since 1998 (Symons et al., 2000). Here, we report the results of our latest survey which include data from other countries including Iran. Since in our survey we did not detect the virus in Iran, we propose a scenario on the origin of this virus as predicted by Meng et al. (2006) (see also Terlizzi et al, 2011).

MATERIALS AND METHODS

Samples from overseas were either sent as canes (only from New Zealand) or as extracts in 4 M guanidine hydrochloride in Na-acetate, pH 5.0 (Mackenzie's buffer). Samples from Iran were collected by the author during 2001-2013, unless specified. These Iranian samples were from four provinces of Khuzestan in the south west, Fars (from 8 different locations collected by A. Afsharifar) in the south, Lorestan in the west and Kashan in the centre. All the samples were from native white or red table grape varieties which were growing on their own roots. Samples from Kashan (Abyaneh) were from a single vine of nearly 200 years old growing in a shrine.

Total nucleic acids were extracted from the phloem shavings of dormant canes essentially as described by MacKenzie et al. (1997). For virus detection, single tube RT-PCR was performed using the primer pair RSP48 (5'AGCTGGGATTATAAGGGAGGT) and RSP49 (5'CCAGCCGTTCCACC ACTAAT) which produced an amplicon of 329 bp from the CP gene (Zhang et al., 1998). Amplicons primed with RSP 48/RSP49 were *cloned into* the *pGEM-T Easy vector* and sequenced using universal M13 forward or reverse primers (AGRF, Adelaide, Australia).

RESULTS AND DISCUSSION

In our earlier survey, we reported that GRSPaV was present in 67.5% of grapevine samples sent to our lab from the viticultural regions of Australia (Habili and Symons, 2000). This has steadily increased to 90.4% in the period 2001-2014 (Table 1). The incidence rate for Germany is second after Australia and stands at 61%. We confirmed the results of biological indexing reported by Goheen (1989), which gave values of 67% and 42% for Australia and Germany, respectively (Table 1).

A very high rate of virus infection was also obtained from Argentina, Thailand, Bulgaria and Israel. An exotic rootstock (Dogridge) planted in India for wine grape grafting was also positive for the virus (tested in 2011). The rate of infection of GRSPaV in Poland is 72% (331 of 460 samples) (Komorowska et al., 2014). GRSPaV has also been detected in Turkey (N. Buzkan, these proceedings).

No sample from any of the four Iranian provinces was tested positive for GRSPaV. A single 200-year old grape in central Iran was only infected with grapevine fleck virus. Other researchers also failed to detect GRSPaV in Iran (K. Izadpanah, pers comm). Since ancient times, the main purpose of grape growing in Iran has been for dried fruit and fresh table grape consumption. The growers only plant locally grown varieties on their own roots. However, in most other countries, including India, the main purpose of viticulture is for wine making. Meng et al. (2006) classified GRSPaV into four major sequence groups of which Group 1 (see also Terlizzi et al., 2011), represented by GRSPaV-1 which is adapted to *Vitis riparia* and group 2 (GRSPaV-SG1) which is adapted to *V. rupestris* are the most common groups. *Vitis riparia* and *V. rupestris* are two species native to North America and carry the virus latently. When the pest Phylloxera (*Daktulosphaira vitifoliae*) accidentally entered Europe in 1860's and devastated vast vineyards, the only solution to stop the pest was to plant *V. vinifera* scions on resistant American rootstocks and their hybrids. Therefore, the virus was introduced with contaminated rootstocks to the European grapevines.

Table 1. Occurrence of GRSPaV in various countries studied in our survey (2001-2014) and its comparison with the biological indexing (Goheen, 1989)

Country	Years	Total tested	Total positive	% +positive	% positive by biological indexing
Australia (a)	1997-2000	2479	1673	67.5[1]	67 (Goheen, 1989)
Australia (b)	2001-2008	4052	3016	74.4	ND[2]
Australia (c)	2008-2014	2215	2003	90.4	ND
Germany	2006-2014	497	304	61.1	42 (Goheen, 1989) [3]
New Zealand	2000-2005	786	278	35.3	ND
USA	2000-2007	83	28	33.7	9 (Goheen, 1989) [4]
Egypt		50	13	26.0	ND
South Africa	2002-2014	824	119	14.4	ND
Iran	2007-2013	66	0	0	ND

[1]Data from Habili & Symons (2000). [2]ND, not determined. [3]The value was 66% for France. [4]Indexing was only for the Foundation Vineyard at Davis (USA).

Meng et al (2006) have found that the virus variants in the American rootstocks are more homogenous in sequence than their counterparts in *V. vinifera* growing in Europe. They concluded that the great heterogeneity of GRSPAV in Europe was caused by high volume grafting following the Phylloxera outbreak. This may have given the chance to the other two sequence groups to emerge, although these are less common, and inter-strain super-protection immunity may have aided diversification (Adrian Gibbs, pers comm).

Old Persia (Iran), being geographically close to the centre of grapevine domestication (Myles et al., 2011) is believed to be the 'centre of emergence' for several grapevine viruses (Hewitt, 1970), but possibly not GRSPaV. It remains to be seen if *V. sylvestris*, if present in Iran, is infected with GRSPaV. This species is believed to be the putative ancestor of the grapevine and it hosts one of the four groups (GRSPaV-VS) of the virus variants.

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OP 36 - Viromes of Hungarian grapevine plantations by next generation sequencing of small RNAs

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INTRODUCTION

As a perennial woody plant propagated vegetatively, grapevine is a potential target of more than 60 viruses and viroids (Martelli, 2014). Viral diseases cannot be controlled by traditional plant protection methods, lifespan of grapevine plantations are greatly influenced by their possible infection with viruses and viroids. Diagnostic methods to detect these pathogens have pivotal role because infections can remain latent for a long period of time. Climate changes and infections with other pathogens can deteriorate the health of the plant resulting enhanced virus activity, causing loss in crop yields, decline and even fall of the plantation. Next generation sequencing methods and the discovery of RNA interference opened new possibilities in virus diagnostics (Navarro et al., 2009, Giampetruzzi et al., 2012, Pantaleo et al., 2010). During virus infection virus originated small interfering RNAs (21-25nt long) representing the exact sequence of the infecting viruses are formed by the plant immune system (Baulcombe, 2004). Deep sequencing and bioinformatics analysis of the small RNA population extracted directly from field grown plants offers a unique opportunity to reveal any virus or viroid present in the sample (Coetzee et al., 2010, Kreuze et al., 2009) even if they were not described before (Giampetruzzi et al., 2012, Wu et al., 2012). In our work we used these cutting edge techniques to reveal sanitary status of grapevine plantations in our country.

MATERIALS AND METHODS

Plant material, sample preparation

During our survey samples were collected from 20 vineyards representing 11 different varieties and 9 wine growing-regions at different locations of Hungary. RNA was extracted from different parts (leaves, shoot tips, flowers and tendrils) of 10 randomly chosen individual plants per plantation by CTAB method (Gambino et al., 2008). Small RNA fraction was purified from plantation pools and used for small RNA library preparation by Illumina TruSeq Small RNA Sample Preparation protocol and sequenced using the Illumina platform.

Pipeline for data evaluation of NGS results (bioinformatics)

Adapters of the sequenced reads were removed and remaining sequences without removing of grapevine specific sequences but without redundancy were aligned to the reference sequences of plant viruses and viroids obtained from the RefSeq database using the BWA short read aligner *aln* method (Li & Durbin, 2009). This non-redundant dataset was applied in the de novo assembly of small RNAs using the Velvet assembler with kmer sizes 13, and 15 (Zerbino & Birney, 2008). The presence of known grapevine-infecting viruses was determined by BLAST search of the assembled short contigs against reference genomes of known viruses. Only hits, with a minimum 200 reads/virus and resulting from at least two independent methods were counted. Distribution of the reads on viral genomes was investigated in order to filter out false positives. Redundant reads of the resulted hits were equalized to read/million read.

Validation of predicted virus diagnostics by RT-PCR

cDNA was synthetized from RNAs representing plantation pools and used as templates for PCR reactions to confirm the presence of the grapevine viruses. For virus detection we used published diagnostic primers for the following viruses:

GLRaV1-3, ArMV, GFKV, GFLV, GVA, GVB and RSPaV (Gambino & Gribaudo, 2006), GCMV (Digiaro et al., 2007), GSyV1 (Al Rwahnih et al., 2009) and GPGV (Glasa et al., 2014). PCR products were analysed by agarose gel electrophoresis and identified according to their size and in some cases by traditional Sanger sequencing (in this case we used individual RNA extracts instead of pools for cDNA synthesis).

RESULTS AND DISCUSSION

Bioinformatics analysis of small RNA sequence reads and its validation by RT-PCR made us possible to get a deep insight into the virus infection status of Hungarian grapevine plantations. Plantation pools instead of samples from symptomatic plants offer an alternative, unbiased way to reveal the presence of viral pathogens in our vineyards. In most cases wet laboratory techniques could verify our bioinformatics results and have shown that the tested plantations are free from GFLV, ArMV and GLRaV-2, -4 and -5. GCMV was present at one, GLRaV3 and GVB at two, while GVA at 5 places. The most widespread infection was found unexpectedly for GPGV at 17 locations. In the case of GFkV and RSPaV RT-PCR was too sensitive and the positive signal failed to coincide with high virus specific read/million read in the sample. Using RT-PCR we detected 18 and 20 infected plantations for GFkV and RSPaV while with bioinformatics methods we have found only 13 and 3 infected plantations, respectively. As a contrast PCR seemed not sensitive enough to detect all GLRaV1 and GSyV1 infection as we showed their presence in 2 and 10 places, while with bioinformatics methods we have predicted them in 8 and 15 plantations, respectively. SNPs in different strains of the viruses located in the used PCR primers or very effective silencing against the viral genomes can both explain this contradiction. The confirmation of viroid infection using RT-PCR will show whether HSVd and GYVd-1 are the most widespread viroids as predicted in the majority (19 and 16, respectively) of Hungarian grapevine plantations. There are still a lot of questions to be solved, but combination of next generation sequencing, bioinformatics and molecular biology provide a powerful new high throughput diagnostic tool to monitor plantations and detect those viruses, which are known to be present in our country and invading pathogens as well. Using this approach we were able to identify several important grapevine infecting viruses, which were not reported from Hungary before.

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OP 37 - Nanobody-based products as diagnostic tool for Grapevine fanleaf degeneration viruses

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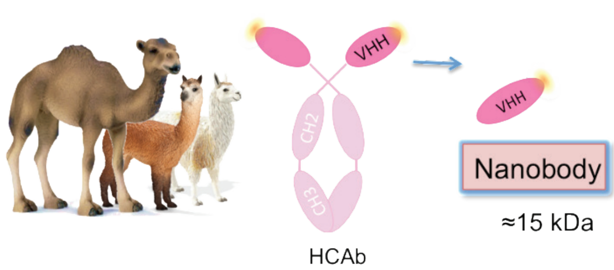
INTRODUCTION

Grapevine fanleaf virus (GFLV) and *Arabis mosaic virus* (ArMV) are the major pathogens responsible for grapevine fanleaf degeneration, one of the most severe viral diseases of grapevine worldwide. These viruses cause substantial crop losses, reduce fruit quality and shorten longevity of grapevine. They are specifically acquired from and transmitted to grapevine by soil-borne nematodes during their feeding process on the growing roots (Andret-Link *et al*., 2004).

No natural resistance to these viruses has been reported so far in *Vitis* species. Since the ban on nematicides to control nematode populations, methods to limit the spread of the disease are restricted to uprooting of grapevine in infected plots, fallow of soil for multiple years and by replanting certified healthy propagative material. Rigorous certification schemes have resulted in significant progress in controlling the spread of GFLV and ArMV through systematic and reliable diagnosis of grapevine propagation material (Oliver and Fuchs, 2011)

The certification of propagative material is mainly based on enzyme-linked immunosorbent assay (DAS-ELISA), using immunochemical reagents derived from polyclonal or monoclonal antibodies originally obtained from mammals after immunization. Their production is expensive and requires very specific structures and skills. Furthermore, the amount and the quality of antibodies produced can be prone to variations in performance. We hypothesized that classical ELISA reagents could favorably be replaced by Nanobodies (Nbs) for the detection of GFLV and ArMV.



Nanobodies are small peptides derived from heavy chain only antibodies found in camelids (Fig.1) (Muyldermans, 2013). They are the smallest naturally occurring intact antigen-binding domains known to date. They are monovalent, stable, soluble, and recognize cryptic epitopes inaccessible to common antibodies. They can be easily tailored and produced to almost unlimited amounts in bacteria such as *E. coli*.

Three complementarity-determining regions (CDR) form the antigen-binding paratope and the sequence of the largest one (CDR3) defines nanobodies families.

Figure 1. Nanobodies derived from heavy-chain-only antibodies.

MATERIALS AND METHODS

Virus isolate and virus purification: GFLV-F13 and ArMV-S were isolated from naturally infected grapevines (Andret-Link *et al.,* 2004). The viral particles were precipitated from clarified *C. quinoa* infected crude sap. Contaminating proteins were removed by centrifugation on a sucrose cushion and two consecutive sucrose density gradients (Schellenberger *et al.,* 2011).

Nanobodies production: *Camelidae* were immunized with purified virus particles at weekly intervals for 6 weeks. GFLV or ArMV specific single domain antibodies (Nbs) or (VHH) were generated according to Ghassabeh *et al.,* 2010. The resulting VHH libraries were screened by phage display for virus-specific binders. Nbs were tailored with appropriate tags using standard molecular biology protocols. Large-scale production of Nbs was performed by expression in *E. coli* and soluble Nbs further purified to homogeneity by affinity and size exclusion chromatography.

DAS-ELISA assessment of Nbs reactivity: virus detection was performed in infected grapevine clarified extracts by DAS-ELISA using anti-GFLV or anti-ArMV IgG as capture antibodies and the tagged Nbs as detection antibodies. For the coating step, tailored Nbs were used as capture antibodies and anti-GFLV IgG, anti-ArMV IgG or tailored Nbs as detection antibodies. Negative control consisted of healthy plants.

RESULTS AND DISCUSSION

23 Nanobodies belonging to 11 families able to recognize GFLV-F13 isolate were produced. To evaluate their ability to detect GFLV in grapevine crude extracts, all anti-GFLV Nbs were tagged. 20 out of the 23 tailored Nbs were successfully produced in *E. coli* and purified to homogeneity as shown in figure 2.

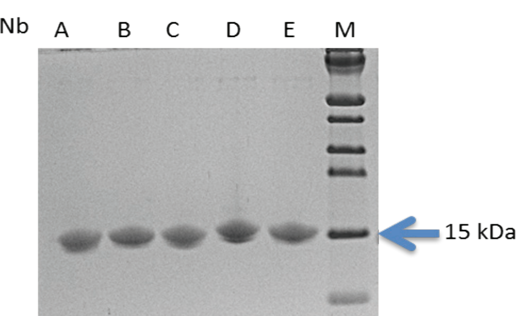


Figure 2: SDS-PAGE analysis of 6 out of the 20 purified tagged Nanobodies. One µg from each Nb after the purification process was separated by SDS-PAGE and stained with Coomassie blue. M: Ladder

The 20 anti-GFLV nanobodies were able to detect GFLV isolates from the grapevine collections of INRA Colmar and IFV Grau-du-Roi. Nanobodies exhibited different spectra according to their family. Importantly, two of them were sufficient to detect all available GFLV isolates (table 1). Our results indicate that Nanobodies are suitable to detect GFLV and can substitute conventional detection antibodies in DAS-ELISA.

	B865	N88	N176	N844	N845	C131	T57	T58	T59	T60	BY47	KyM5	H126	H125
ELISA	++	+	+	+	++	++	++	++	+	++	+	++	+	+
Nb 1A	++	+	+	+	++	++	+	+	+	+	-	++	+	+
Nb 3A	+	-	+	-	++	++	++	-	-	++	++	-	+	+

	N210	BE411	BE519	BE539	B844	CO1	CO2	CO3	CO4	C8619	C8673	Moet	F13	GHu
ELISA	+	++	++	++	+	+	+	+++	+++	++	+	+++	+++	+++
Nb 1A	+	+	+	+	+	+	++	++	+++	+	+	+++	+++	+++
Nb 3A	-	-	-	++	+	+	+	++	++	++	++	+++	+++	-

Table 1: Recognition spectrum of Nbs 1A and 3A in comparison to anti-GFLV conventional antibodies. Green and red colors correspond to positive and negative GFLV detection, respectively. “+” relates to detection levels. Note that Nb 1A recognizes all 28 GFLV isolates except BY47 that is recognized by Nb3A.

A similar approach was initiated to develop reagents for *Arabis mosaic virus* (ArMV) detection, another nepovirus responsible for fanleaf degeneration.

The performance of DAS-ELISA tests using only Nanobody-derived reagents for virus GFLV and ArMV detection from infected leaves and woody material will be presented.

ACKNOWLEDGMENTS

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OP 38 - Occurrence of Grapevine viruses on the South of Russia

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INTRODUCTION

Grapevine viruses are widely occurring on the south of Russia. Symptoms are variable between grapevine cultivars. However viral infections often occur without any symptoms that contributes their wide spread.

Viral diseases negatively affect flowering, shoot and leaves growth. Infected shoots have short internodes, abnormal branching and deformed leaves. So the economic impact of grapevine viruses is high (Credi R., Babini A.R., 2001).

MATERIALS AND METHODS

Samples were collected from vineyards of Sevastopol, Bahchisarai, Simferopol, Sudak, Alushta and Yalta regions. RNA was extracted according to the protocol described Rott and Jelkman (1990). Viruses were tested by RT-PCR with specific primers followed by sequence of PCR-products.

RESULTS AND DISCUSSION

The results of diagnostics showed infection with the most common grapevine viruses: Grapevine rupestris stem pitting-associated virus (RSPaV), Grapevine fanleaf virus (GFLV), Grapevine virus A (GVA), Grapevine leafroll-associated viruses-1 (GLRaV-1). Several samples had mixed viral infection. Grapevine virus B (GVB) and GLRaV-2 and GLRaV-3 were not detected.

ACKNOWLEDGMENTS

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OP 39 - Effect of latent GVB infection on agronomic and enological performances of wine cultivar Albarossa (*Vitis vinifera* L.)

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INTRODUCTION

Grapevine virus B (GVB), member of the genus *Vitivirus*, is closely associated with Corky bark disorder of the Rugose Wood complex. Although not particularly widespread in grape, it is generally considered harmful and the Italian regulations (DM 24/06/2008) listed the GVB among the viruses from which a selected clone must be free to be registered in the National Catalogue. Few, if none, information however were reported on the real impact of latent GVB infection in grapevine. The aim of this study was to ascertain agronomic and enological performances of symptomless vines affected by GVB in comparison with GVB-free plants.

MATERIALS AND METHODS

A symptomless clone of the wine red cultivar Albarossa (*Vitis vinifera* L.) tested free from GFLV, ArMV, GLRaV-1, GLRaV-2, GLRaV-3, GVA and GFkV, induced Corky bark-like symptoms on woody indicator LN 33 and the subsequent mRT-PCR analysis (Gambino and Gribaudo, 2006) detected the presence of GVB and GRSPaV. Afterwards a GVB-free clonal line was produced through *in vitro* meristem culture. Cuttings collected from originally GVB-infected (+GVB) and GVB-free (-GVB) mother plants were rooted and kept in collection in a screen-house. In 2009, scions collected from the screen-house plants were propagated by grafting onto healthy Kober 5 BB rootstock. The following year 16 vines per each thesis were planted in a commercial vineyard arranged in four replicates of 4 vines each along two parallel rows. The vineyard was located in a typical area for Albarossa cultivation in North-West Italy and vines were vertically trained and single cane pruned with a density of 3500 vines/hectare. The virological status of every single vine in the trial was controlled on dormant cane samples collected during 2013-14 winter by DAS-ELISA (Agritest srl, Valenzano, Italy and Sediag INRA, France) and by mRT-PCR (Gambino and Gribaudo, 2006). The tests confirmed that all the +GVB plants had the virological status originally detected, i.e the plants, although symptomless, were GVB and GRSPaV infected and free from GFLV, ArMV, GLRaV-1, GLRaV-2, GLRaV-3, GVA and GFkV, whereas all the -GVB plants were free from all the above mentioned viruses except for GRSPaV. In the 2014 season, when the vines were adult and fully productive, biometric, agronomic and juice qualitative parameters were assessed on a four replicates basis. Field data were statistically elaborated by ANOVA. The total amount of harvested grapes of both +GVB and -GVB vines was separately submitted to small scale winemaking. Chemical and sensory evaluations were then performed on the wines a few months after bottling. Sensory evaluation was carried out by an expert panel of 13 tasters using a ‘duo-trio’ tasting test (i.e. the panelist must pick out the two identical wines among a group of three) followed by a paired-preference test. A characterization test was then carry out to investigate the intensity of different components of color, bouquet and taste.

RESULTS AND DISCUSSION

The elimination of GVB induced a significant increase of plant vigor and yield (Table 1). The superior yield was mainly due to a higher number of cluster/vine. The cluster weight did not vary much between healthy and infected plants however in the former the berries resulted smaller (and supposedly higher in number per cluster), meaning an improved fruit setting. Despite the 1 kg per vine yield increase performed by GVB-free plants, the juice sugars concentration was unaffected and the juice acidic parameters were even reduced, particularly the amount of tartaric acid. In terms of enological quality, however, chemical data showed that the wine from -GVB vines had a slightly lighter composition compared to wine from +GVB plants (Table 2). This was not surprising in consideration of the higher yield produced by healthy plants which resulted in a wine with lower degrees of alcohol, dry total extract and acidy. In addition the wine of GVB-free plants was lower in total anthocyanins (responsible of wine color) and total flavonoids (responsible of wine body). Differences in grape phenol extraction depend by the evolution of phenols during grape ripening which is clearly influenced by grape yield. The higher amount of total anthocyanins and total flavonoids in the wine from +GVB vines, as well as the higher acidity, had consequently some important positive sensorial effects well evidenced by wine tasting results. Thanks to the higher

content in anthocyanins, the color of this wine was preferred by all the panelists when compared to -GVB wine (Figure 1). The characterization test clearly confirmed the +GVB wine was characterized by a higher color intensity with a more evident violet nuance (Figure 2). Likewise, the panelists evidenced a stronger body, a longer taste persistence and bitter aftertaste, all parameters influenced by the flavonoids content. Although in terms of bouquet and taste the panel did not give a clear preference to one or to the other wine, the overall judgement of the majority of tasters (9 to 4) was in favor of the wine obtained by GVB-infected vines thanks to the better color (Figure 1). In conclusion, the results indicate that the elimination of GVB from symptomless vines induced a clear enhancement of field performances (vigor and yield) without beneficial effects on enological quality. Similar results due to the increase of yield in the first years of the vineyard life often associated to virus sanitation were already reported in previous experiences (Mannini *et al*.,2010).

Table 1 – Field data of GVB-free and GVB infected Albarossa vines. n.s.= not significant, *, **, ***= significant at p≤0.05, 0.01 and 0.001, respectively.

FIELD DATA	*GVB-free*	*GVB*	*F*
Fertility (n° inf/shoot)	1.93	1.80	ns
Yield (kg/vine)	3.91	2.91	*
Cluster/vine (n°)	14	11	*
Cluster wt (g)	279	275	ns
Cluster ln (cm)	18	16	***
Cluster wd (cm)	13	13	ns
Berry wt (g)	1.53	1.67	*
Berry ln (cm)	1.45	1.50	*
Berry wd (cm)	1.25	1.30	*
Pruning wood wt (g/vine)	650	570	**
Soluble solids (g/L)	22.0	22.2	ns
Titratratable acidity (g/L)	11.78	12.37	ns
pH	2.81	2.79	ns
Tartaric acid (g/L)	10.0	10.6	*
Malic acid (g/L)	3.12	3.30	ns

Table 2 – Wines composition of GVB-free and GVB-infected Albarossa vines.

WINE DATA	*GVB-free*	*GVB*
Alcohol (% vol)	12.85	13.12
Dry extract (g/L)	28.9	30.5
Titratable acidity (g/L)	8.86	9.42
pH	3.19	3.15
Tartaric acid (g/L)	2.02	2.31
Lattic acid (g/L)	2.44	2.55
Potassium (mg/L)	1024	1031
Ash(g/L)	2.89	2.97
Total flavonoids (mg/L)	1226	1493
Total anthocyanins (mg/L)	381	446
Color intensity (A420+520+620)	11	13
Color hue (A420/520)	0.52	0.48

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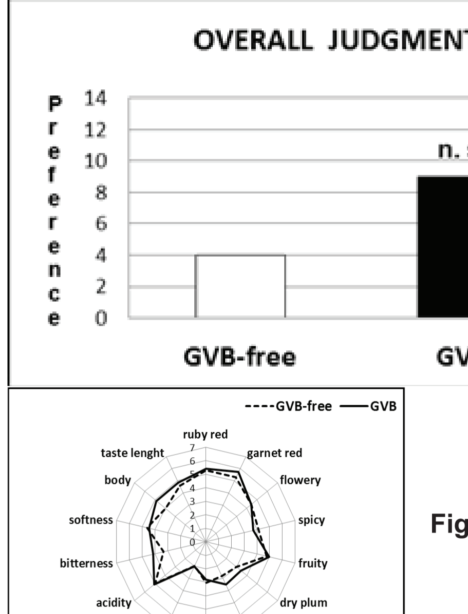
Figure 1 – Paired preference test on Albarossa wines obtained by GVB-free and GVB-infected vines. Panel: 13 tasters, n.s.= not significant, *=significant at p≤0.05.



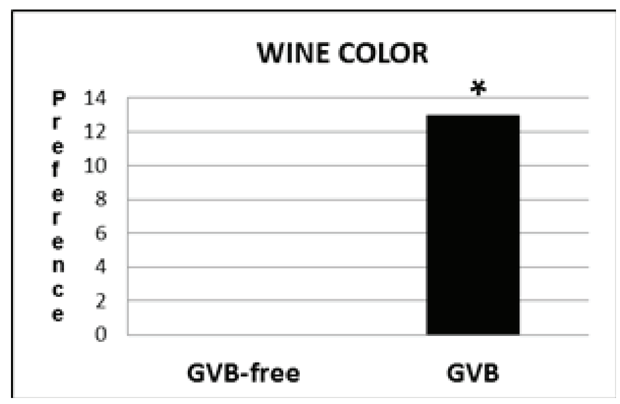


Figure 2 – Sensory profile of Albarossa wines of GVB-free and GVB-infected vines)

OP 40 - Searching for the needle in a haystack: Small RNA analysis of grapevine leafroll disease in symptomatic and asymptomatic cultivars

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INTRODUCTION

Small non-coding RNA (sRNA), which can range from 20 to 24 nucleotides, are involved in a number of plant activities varying from normal development to biotic and abiotic stress responses (Axtell, 2013a). Small RNAs regulate numerous biological processes by interfering with mRNA translation or by initiating the cleavage and subsequent degradation of mRNA (Ruiz-Ferrer and Voinnet, 2009). In plants, sRNAs can be divided into microRNAs (miRNAs) and small interfering RNAs (siRNAs) based on their biogenesis and function (Khraiwesh et al., 2012). Plant pathogens can activate RNA silencing through different mechanisms including production of their own sRNAs or by altering endogenous sRNA levels in plant hosts (Ruiz-Ferrer and Voinnet, 2009). In this study we aimed to use next-generation sequencing (NGS) to construct sRNA profiles to characterise the plant response to *grapevine leafroll-associated virus* 3 (GLRaV-3) infection. We compared sRNA profiles of symptomatic versus asymptomatic GLRaV-3 infected grapevine cultivars to elucidate possible virus-host interactions that may contribute to host symptom expression and specificity.

MATERIALS AND METHODS

Six young plants of cultivars Chardonnay, Chenin blanc and Cabernet Sauvignon were collected from a certified virus-free nursery and re-established in the greenhouse. *Grapevine leafroll-associated virus* 3 variant group II were graft inoculated onto three plants from each cultivar using infected buds. Phloem material was sampled from all plants in the same physiological growth stage as soon as the shoot material reached lignification (Figure 1). High quality total RNA was extracted from phloem material using a modified Cetyltrimethylammonium bromide (CTAB) protocol (Carra et al., 2007) and the quality was assessed using the Agilent Bioanalyser. The virus status of these plants was confirmed using end-point RT-PCRs for frequently occurring grapevine viruses and GLRaV-3 virus titre was determined using an RT-qPCR assay (Bester et al., 2014). A sequencing library was prepared from total RNA for each plant sample using the Illumina Small RNA TruSeq kit and sequenced on an Illumina HiSeq. Adapter sequences were removed and reads were filtered for quality (phred score >20) using Fastx toolkit. Only reads 18 - 26 nt in length were used for sRNA analysis. Virus-derived siRNA (vsiRNA) reads were identified by mapping to a reference genome (GLRaV-3, isolate GP18, EU259806). Bowtie (1.0.1) (Langmead et al., 2009) was used to perform all read-mapping analyses. Known *Vitis vinifera* miRNAs as well as sRNAs homologous to known miRNAs of other plant species were identified using in-house scripts, allowing no mismatches to entries in miRBase version 21 (Kozomara and Griffiths-Jones, 2014). Shortstack (v2.0.9) (Axtell, 2013b) was used to perform novel miRNA prediction from sRNAs mapped with no mismatches to the *Vitis vinifera* genome. Potential phased regions in the *Vitis vinifera* genome and transcriptome were identified using ShortStack. Potential phase-initiating miRNAs were identified using the web-based tool, psRNATarget (Dai and Zhao, 2011). To determine variation in sRNA expression levels between the healthy and the GLRaV-3-infected samples, the R package, DESeq2 (Love et al., 2014) was used. Targets for differentially expressed sRNAs were predicted using psRNATarget. BLAST homology searches were performed using Blast2GO (Conesa and Götz, 2008).

RESULTS AND DISCUSSION

An average of seven million high quality reads between 18 to 26 nt were generated per sample. The libraries were dominated by reads 21 nt in length followed by 24 nt long reads. The 21 nt reads also displayed the greatest redundancy. Small RNAs that often fall into this size group are miRNAs and phased siRNAs (phasiRNAs). Reads, which did not align to the *Vitis vinifera* nuclear, chloroplast or mitochondrial genomes, were mapped onto the complete genomes of 13 GLRaV-3 isolates. Read mappings identified virus-derived siRNAs (vsiRNAs) originating from GLRaV-3 variant group II. These variant-specific reads were distributed along the length of isolate GP18 (variant group II) confirming the presence of this variant in infected samples.

Reads with perfect matches to *Vitis vinifera* miRNAs (vvi-miRNAs) in miRBase (version 21) were classified as known miRNAs. Sixty eight percent of the 119 unique vvi-miRNA sequences in miRBase could be detected in the three different cultivars. The miRNA cluster with the highest read count, vvi-miRNA166, accounted for 74% of all the reads mapped to vvi-miRNAs. The vvi-miR166 family is predicted to target *Vitis vinifera* homeobox-leucine zipper proteins, which are involved

in a range of plant processes including plant growth and development (Elhiti and Stasolla, 2009). Furthermore, 3.5% of the total reads mapped with 100% identity to miRNAs from other plant species in miRBase. In order to consider these miRNAs as vvi-miRNAs, their region of origin on the grapevine genome were analysed during novel miRNA prediction. For the Chardonnay, Chenin blanc and Cabernet Sauvignon libraries, 137, 126 and 138 clusters were predicted, respectively. Only 44% of these clusters overlapped with known hairpin loci.

Differentially expressed miRNAs between diseased and healthy samples of known and novel origin were identified for both Chardonnay and Chenin blanc. Even though different miRNAs were differentially expressed between cultivars, these miRNAs are predicted to target similar genes including growth-regulating factors, homeobox-leucine zipper proteins, NADPH-dependent diflavin oxidoreductase and pentatricopeptide repeat-containing proteins. All of which can be involved in the biotic stress responses in grapevine.

Shortstack also predicted clusters of sRNAs to originate from both genomic and transcript phased loci. Potential miRNA phase-initiators were identified for these loci. After examining the phased loci using BLAST analysis the majority of these aligned against disease resistance proteins, followed by MYB transcription factors and pentatricopeptide repeat-containing proteins.

These findings provide resources for further development to elucidate the complex host-pathogen interactions at play in grapevine leafroll disease and can contribute to understanding the unknown mechanisms of GLRaV-3 pathogenicity in symptomatic cultivars.

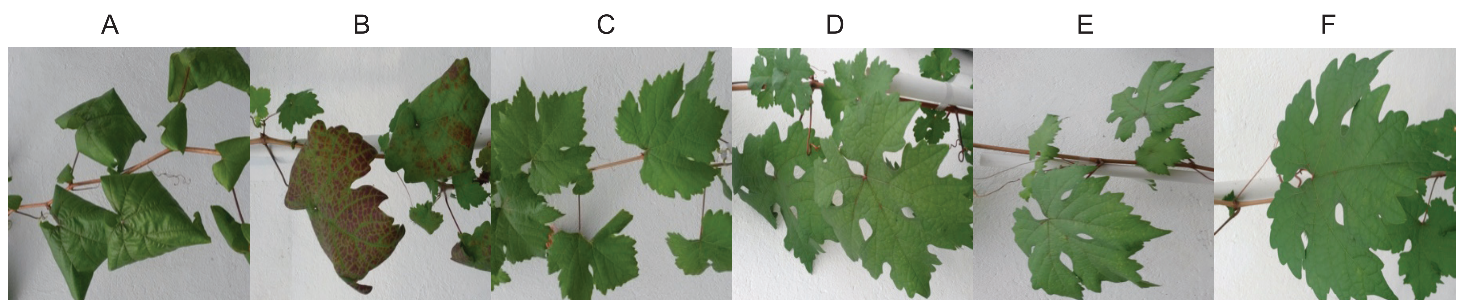

Figure 1. After lignification of the primary shoot, clear GLRaV-3 symptom expression was observed in infected Chardonnay (A) and Cabernet Sauvignon (E) plants compared to the infected Chenin blanc (C) and healthy Chardonnay (B), Chenin blanc and Cabernet Sauvignon (F) plants.

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OP 41 - Differential gene expression profiling of grapevine cultivars Touriga Nacional and Cabernet Sauvignon infected with *Grapevine leafroll-associated virus 3* (*GLRaV-3*) and *Grapevine fanleaf virus* (*GFLV*)

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INTRODUCTION

Grapevine leafroll disease (GLD) is the most important disease of grapevines, occurring in every grape-growing country (Martelli and Boudon-Padieu 2006). GLRaV-3 is the type member and the most widespread of the Ampeloviruses implicated in GLD. In turn, infectious degeneration, another most relevant and widespread grapevine virus diseases (Raski *et al.*, 1983; Bovey *et al.*, 1990; Martelli and Savino, 1990), is mainly induced by distorting and chromogenic strains of the Nepovirus grapevine fanleaf virus (GFLV). Both are certification viruses in the EU. The yield and life span of a vineyard can be compromised by the degree of susceptibility of the scion and/or rootstock to those diseases.

Beyond the description of symptoms associated to each disease or causative agent, and after the sequencing of the grapevine genome, surveys of virus-host interactions at the transcriptional level are being conducted. In cultivars susceptible to GLRaV-3 (Espinoza et al., 2007; Gutha et al., 2010) evidence suggests that transcripts associated to cellular function and transport, are mostly repressed. Although natural resistance to virus in grapevine has not yet been demonstrated, it is general knowledge that the extent of the response to a disease is cultivar dependent. In this scenario, the quantification of distinct changes in gene expression, allied to performance description for each of those pathosystems, will permit to identify susceptibility and/or resistance related transcript profiles for cultivar characterization.

Recently a set of differentially expressed genes was identified in our lab for cv. Touriga Nacional, associated to the response to infections by GLRaV-3, GFLV or both viruses. Following this background work, quantification of the expression of these genes was done by qPCR. Identification and testing of reference genes, with stable expression in the systems studied, was conducted for normalization of the qPCR data. Taken together, our results present guidelines for reference gene(s) selection and revealed significant differences between the two types of viral infection in the transcriptional response of these grapevine cultivars. The present study provides a foundation for the selection of several candidate genes for further functional analysis and comparative genomics with other grapevine cultivars.

MATERIALS AND METHODS

Plant material

Plants of the cultivars Touriga Nacional and Cabernet Sauvignon, established in a commercial vineyard, were screened for infection with GFLV and GLRaV-3, using commercial antibodies for DAS-ELISA and also using RT-PCR with virus-specific primers.

Healthy and infected plants were followed for two years and symptoms recorded.

Material for Differentially Expressed Genes (DEGs) analysis was collected from expanding buds during Spring 2012. At least four plants per type of infection and four healthy ones were sampled for each cultivar.

RNA extraction

For each sample, total plant RNA was extracted with an E.Z.N.A.™ Plant RNA Kit (Omega Bio-Tek, USA). Presence of the viruses was verified through RT-PCR with virus-specific primers

DEG analysis

The Seegene GeneFishing™ kit (Seegene, BioGene, UK) was used to detect differentially expressed genes, following

the manufacturer's instructions.

Cloning and sequencing

Differential bands were purified and the amplicons were ligated with the CloneJET™ PCR Cloning Kit (Thermo Fisher Scientific, Inc., USA) and used to transform *E. coli* XL1Blue (Agilent Technologies Inc., USA) competent cells. The positive recombinants were sequenced.

Sequence data analysis

A BAST analysis was conducted for each DEG sequence, using the EST database at GenBank, to allow identification of the DEGs detected.

qPCR

Validation of each DEG detected was done through Real-time PCR quantification (qPCR), using the Relative Standard Curve method. Specific primer pairs for each DEG detected were designed.

For normalization to an endogenous control, three putative housekeeping genes were tested: NAD5, EFα and β-actin. The NormFinder software was used to identify the optimal normalization gene. The geometric mean of the quantified expression of EFα and β-actin was used.

RESULTS AND DISCUSSION

A set of differentially expressed genes (DEGs) was identified in cultivars Touriga Nacional and Cabernet Sauvignon, associated to the response to infections by GLRaV-3, GFLV or both viruses.

Following this background work, quantification of the expression of those DEGs is underway, using qPCR. Preliminary results are shown for both cultivars. (Data shown on poster).

Identification and testing of reference genes, with stable expression in the systems studied, was conducted for normalization of the qPCR data. EFα and β-actin were identified as the most suitable normalization genes for this cultivar.

The present study provides a foundation for the selection of several candidate genes for further functional analysis and comparative genomics with other grapevine cultivars.

ACKNOWLEDGMENTS

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OP 42 - Grapevine leafroll disease spread from old to replacement vineyards

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INTRODUCTION

Grapevine leafroll disease is associated primarily with infection by grapevine leafroll-associated virus 3 (GLRaV-3) (Pietersen et al., 2013) and spread mainly by *Planococcus ficus* (vine mealybug) (Krüger and Douglas-Smit, 2013) in South Africa. The possibility that leafroll infection can spread from an old vineyard to a young, replacement vineyard on the same site (Pietersen, 2004) was based on; 1) the demonstrated subterranean phase in the lifecycle of *P. ficus* (Walton and Pringle, 2004), 2) recording of considerably lower levels of leafroll in the half of a commercial vineyard subjected to a fallow period (Pietersen et al., 2006), 3) persistence of GLRaV-3 in remnant roots and the discovery of *Pseudococcus calceolariae* on remnant roots six to 12 months after herbicide treatment (Bell *et al*., 2009), and 4) the common occurrence of leafroll infected volunteer vines from old vineyards. In this study we intend to confirm this mode of spread as well as assessing various methods to prevent it from occurring. We evaluated fallow periods of differing duration during which time infected remnant vine material was removed, the use of systemic insecticides prior to vineyard removal, and the use of herbicides to prevent the persistence of living *Vitis* material from a preceding vineyard.

MATERIALS AND METHODS

The trial site was a commercial vineyard of 6466 vines planted in 1982 to *Cabernet sauvignon* in Paarl (S33°43.105 E19°00.887). It was completely infected with leafroll disease, with representative samples all containing GLRaV-3. It contained a ubiquitous infestation of *P. ficus* for at least two seasons prior to treatments. The lat/long co-ordinates of all replicated blocks, pathways and surrounds of the trial site were determined with a differential GPS with post processing. The position of leafroll infected vines within the newly established vineyard was recorded using the same GPS and correlated with the treatment applied in the preceding vineyard. Fallow treatments consisted of removal of vines from three replicated blocks of twenty plants by ten rows (200 vines) in a randomized split-plot design. Vines were mechanically removed. Blocks were kept fallow for either, one, two or three growth seasons during which time volunteer plants were removed. All vines in the replicated (n = 3) 10 rows by 20 vine blocks requiring herbicide treatment were treated with a 10% glyphosate (Roundup®, Monsanto, USA) cut-stump application during spring (October) 2007. Herbicide treated vines were mechanically removed in the subsequent winter. Imidachloprid (Confidor® 350SC, Bayer, Germany) was applied to all vines in spring (September) 2007 in the replicated (n = 3) blocks of 10 rows by 20 vines. Application was by soil drench around the base of stems with 1.5ml product per vine in 100ml water followed by at least 4 litres of water. Actual treatment plots were positioned to have buffer zones of at least ten vineyard rows from adjoining vineyards or the edge of the vineyard. Surrounding vineyards were removed during the course of the trial and healthy vineyards established on these sites. All remaining old vines in the trial (controls) were removed in autumn (May), 2008, and the entire site prepared for a new vineyard using standard industry practices. New vines were established in the summer (December) 2009. Healthy nuclear US 8-7 rootstock material testing free of GLRaV-3 was established from rooted canes. Those that did not take were replaced within two months. Nuclear material of *Cabernet franc* 1J, individually testing negative for GLRaV-3 was field grafted onto the rootstocks in late November, 2010. Vine-for-vine inspection was done annually in autumn in the new vineyard and all vines tested by ELISA for GLRaV-1, -2, and -3. GLRaV-3 infected vines were treated with imidachloprid in spring of the season directly after they were detected. Volunteer vines in vineyards and feral vines growing in the proximity of the new vineyard were removed annually.

RESULTS

The number of leafroll infected vines observed annually within the new vineyard correlating spatially with the treatments conducted in the old vineyard is presented in Table 1.

Table 1: Number of grapevine leafroll infected plants within treatment replicates for 2011, 2012 and 2013.

Treatment	Replicate	Number of leafroll infected plants		
		2011	2012	2013
Fallow- one season	1	0	0	1
	2	0	0	1
	3	1	4	15
Fallow- two seasons	1	0	1	4
	2	0	0	2
	3	1	2	4
Fallow- three seasons	1	0	0	0
	2	0	3	7
	3	0	1	4
Herbicide (glyphosate)	1	0	2	5
	2	0	2	6
	3	0	0	1
Imidachloprid	1	0	0	2
	2	3	3	10
	3	1	1	4
Control	1	0	0	1
	2	2	2	4
	3	0	0	4

DISCUSSION

The trial failed unequivocally to confirm the spread of leafroll disease from an old infected vineyard, to a new replacement vineyard on the same site. While eight infected vines were detected within the same season when scions were field grafted and clearly reflect rapid infection in the field, they were distributed amongst the replicates of the various treatments as well as the controls, and therefore the low numbers preclude any meaningful statistical analysis. In the second season, 21 infected vines were obtained, still too low to analyse. While more (n = 76) leafroll infected plants were obtained in the third season (2013), many of these are within one or two plants along a row (and sometime across a row) from a vine infected the previous season. While this may reflect remnant foci of high mealybug numbers from the old vineyard, it may also be due to secondary spread of GLRaV-3. This confounds any data of leafroll plants infected within the treatment site of the previous vineyard, and suggests the failure of the trial. We present the results so that future studies may improve upon the design of such a trial, preferably with larger treatment numbers and more successful secondary spread control.

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OP 43 - Virus incidence and dispersion in the Grapevine Germplasm Bank in Andalucía, Spain

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INTRODUCTION

The Grapevine germplasm bank in Jerez de la Frontera (Cádiz, Spain) was established more than one century ago and hosts nearly two thousand grapevine accessions from all around the world, mainly from Spain. We are currently carrying out a program for the genetic characterization by ampelography and microsatellite markers of the accessions and simultaneously the sanitary status of the plants is being evaluated.

MATERIALS AND METHODS

The bank is split into six parcels averaging 300 entries each, consisting in five plant replicas per entry. The virus sanitary evaluation was performed by DAS-ELISA (Agritest, Italy) for five virus species: GLRaV-1, GLRaV-2, GLRaV-3, GFLV and GFkV. ArMV was not analyzed because its incidence in Spain is residual, although its presence in entries from other countries cannot be discarded. Two plants per entry were analyzed for each virus. To date, two parcels consisting in 149 (parcel #10) and 360 (parcel #8) entries, respectively, have been analyzed. Each box in Figure 1 represents an input of five plants, corresponding to each grapevine entry. The spatial dissemination of GLRaV-1, GLRaV-2 and GFLV in parcel #8 was studied by the binomial and beta-binomial distribution analysis using the BBD software (Madden, 1993). Results can discern whether diseased plants are grouped (clustered) or not. Alternatively, the SADIE analysis of virus dispersion was performed (Li *et al*., 2012). In addition, the presence of nematodes in the soil has been investigated. For that, soil samples at two depths (30 and 60 cm) were taken and analyzed according to Flegg (1967). The incidence of mealybugs has also been studied by using species-specific pheromone traps and multiplex PCR for distinguishing among the Pseudococcidae species (Daane *et al*., 2011).

RESULTS AND DISCUSSION

The presence of nematodes resulted very low, although individuals of genera *Xiphinema*, *Longidorus* and *Amplimerlinius* have been identified. Among the *Xiphinema* nematodes none of them belonged to the species *X. index* or *X. italiae*. Consequently, no plant parasitic nematodes which can transmit harmful viruses are present in the bank.

Table 1: Incidence of the viruses in the parcels studied in the bank.

	GLRaV-1	*GLRaV-3*	*GFLV*	*GLRaV-2*	*GFkV*
Parcel #10 (149 entries)	17.1	92.2	7.8	31.5	40.5
Parcel #8 (360 entries)	18.0	95.9	7.6	38.4	58.5

The spatial analysis of infected plants may help to detect the occurrence of *in situ* virus dispersion (Figure 1). Results show that for GLRaV-2 the beta-binomial distribution in parcel #8 describes better the data than the binomial distribution. This result suggests *in situ* spread of this virus species by some insect vector. This is significant since the natural vector for GLRaV-2 is not clearly identified yet. On the other hand, the SADIE analysis of the GLRaV-2 incidence in parcel #8 also showed a pattern of significant aggregation (Ia = 2.344; P(Ia) = 0.001). It is generally considered if the Ia index > 1 does exist spatial aggregation (Perry & Dixon, 2002). Graphically, the asymmetry of the frequency distribution of distance values obtained regularly during the permutations indicated the presence of significant aggregation (Figure 2).

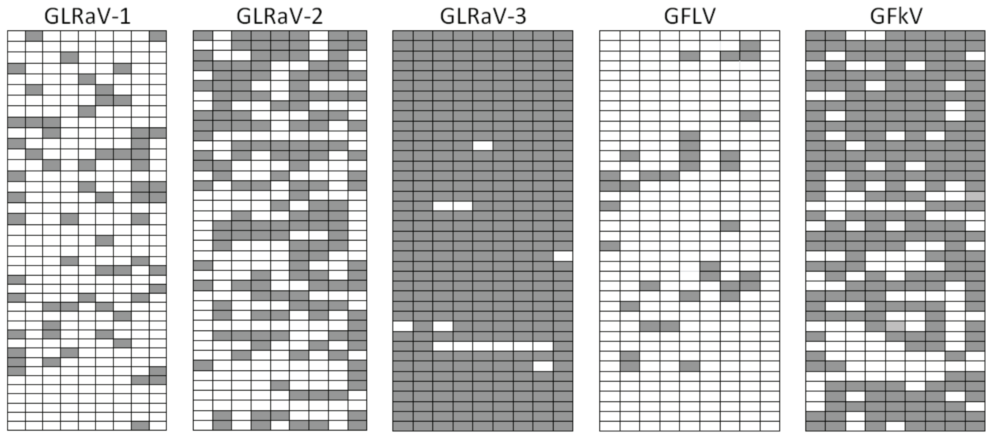

Figure 1: Distribution of the viruses in parcel #8.

Regarding the high incidence of GLRaV-3 in parcel #8 as well in parcel #10, it leads us to suspect the existence of *in situ* infection in the bank. The incidence of GLRaV-3 in both parcels bank was much greater than in conventional vineyards, taking into account the different origin of the vines. The abundant presence of mealybugs, mainly *Planococcus ficus*, an efficient vector for GLRaV-3 transmission, supports this hypothesis (Figure 3). The incidences of GLRaV-1 and GFLV were low as expected. The SADIE analysis of GLRaV-1 dispersion in parcel #8 showed no significant aggregation (Ia = 1.234; P(Ia) = 0.153) and therefore suggests that this virus is not spreading, as occurs with GFLV. Since the dispersion of both diseases is random it implies that there is no dissemination of both diseases in the bank. Finally, the incidence of GFkV was very high, supporting the hypothesis that it is frequently transmitted simultaneously with GLRaV-3 (Cretazzo *et al*., 2010).

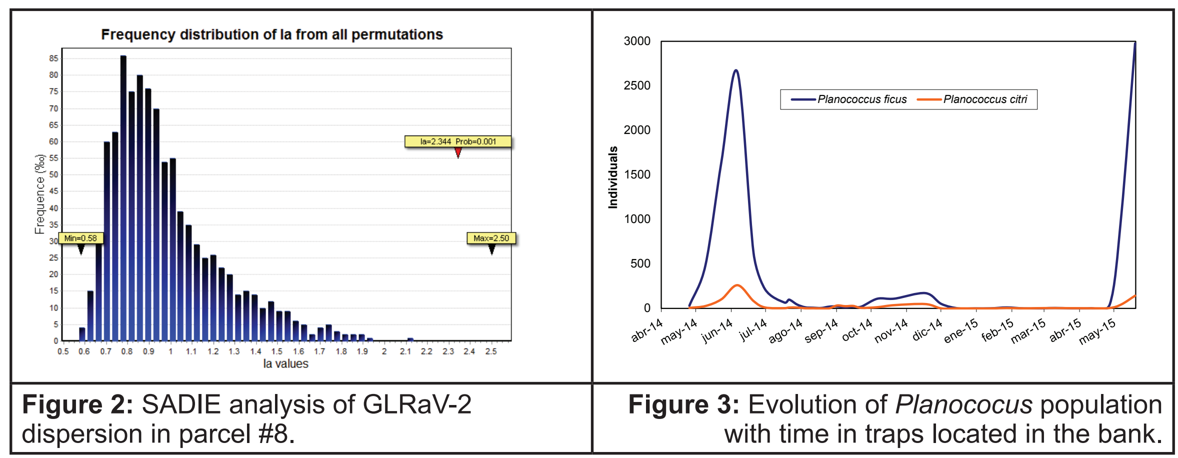

Figure 2: SADIE analysis of GLRaV-2 dispersion in parcel #8.

Figure 3: Evolution of *Planococus* population with time in traps located in the bank.

Currently, we are carrying out the sanitary analysis for the rest of the parcels that will allow to determine the total incidence of viruses in the bank and to confirm the possible *in situ* spread of GLRaV-2.

ACKNOWLEDGMENTS

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OP 44 - Transmission studies of the *Grapevine leafroll-associated virus 1* and the *Grapevine virus A* by the mealybug *Phenacoccus aceris.*

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INTRODUCTION

Grape production faces many severe viral, bacterial and fungal diseases, among which leafroll is one of the most devastating worldwide. Leafroll disease can be caused by several viruses belonging to the *Closteroviridae* family (GLRaV-1, -2, -3, -4 and -4-like). While GLRaV2 (genus *Closterovirus*) has no known vector and to our knowledge does not spread naturally in vineyards, the other three ones (*Ampelovirus*) are specifically transmitted by mealybugs (*Pseudococcidae*) and soft scales (*Coccidae*) and thus prone to be efficiently dispersed within and between vineyards (Sforza *et al*., 2003 ; Le Maguet *et al*., 2013). GLRaV-1 and GVA have flexuous virions of ca. 2000 nm and 800 nm composed of positive ssRNAs of 18,7 Kb and 7,5 Kb, respectively.

GLRaV-1 and GVA are restricted to vascular tissues of host plants and are frequently present in co-infection in grapevine. Their transmission by mealybugs has been shown to comply the criteria of a “semi-persistent and non-circulative” mode (Tsai *et al*., 2008). The mealybug *Phenacoccus aceris* has been shown to transmit very efficiently ampeloviruses and vitiviruses (Le Maguet *et al*., 2012), but neither the biological parameters of the transmission, nor the precise location of virions specific retention within the vector are known so far.

MATERIALS AND METHODS

To address these questions, mouthparts anatomy of the vector *P. aceris* was described using scanning electron microscopy (SEM) and transmission electronic microscopy (TEM) techniques. Transmission tests from infected to healthy plants were developped to determine the biological parameters of the transmission, such as the minimum acquisition and inoculation access periods of the viruses by mealybugs, and the maximum retention time within the vector. Fluorescent labelling by successive membrane feedings were used to locate the precise zone of particles retention. Virions were purified following the protocol described by Gugerli *et al*. (1984) and hybridization with specific primary (BioReba) then secondary (ALEXA Fluor 488 & 568) antibodies was used to label the virions particles within the mealybug.

RESULTS AND DISCUSSION

Phenacoccus aceris mouthparts anatomy appears to be close to those of other sap-sucking insects.

Mouthparts are composed of a clypeus, a labium and the stylet fascicle. The three-segmented labium bearing some mechanosensory hair-like sensilla, show a labial groove on its anterior surface, enclosing the stylet fascicle. The stylet bundle is needle-like and composed of two distinct mandibular and maxillary stylets.

Experiments to determine the biological parameters of the transmission showed that *P. aceris* is able to acquire and transmit both viruses in a very short time (1 h acquisition and inoculation time). However, the transmission efficiency increase in time as previously shown for the GLRaV-3 transmission by *Planococcus ficus* (Tsai *et al*., 2008).

The “semi persistent and non circulative” transmission mode of the GLRaV-1 and the GVA implies a virion retention on ectodermic structures, such as the stylet fascicle and/or the foregut of the insect.

ACKNOWLEDGEMENTS

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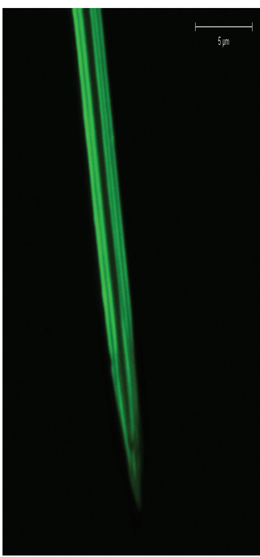

Figure 1. Tip of a P. aceris maxillary stylet in confocal microscopy

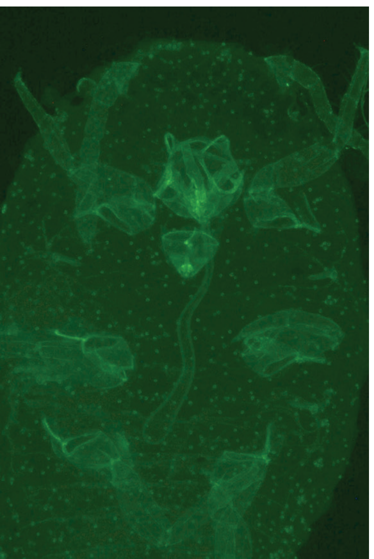

Figure 2. Ventral view of a P. aceris nymph in epifluorescence microscopy

OP 45 - (Invited Lecture) Phytoplasma diseases in grapevine a threat to worldwide viticulture

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HYSTORICAL BACKGROUND

Phytoplasmas are associated with grapevine diseases in the majority of viticultural areas worldwide; symptoms mainly involve plant decline, leaf rolling, shrivelled grapes, unripened shoots and reddening or yellowing of leaves on red or white cultivars respectively. Molecular studies have provided considerable insights into their molecular diversity and genetic interrelationships; taxonomic progress has been achieved by using 16S ribosomal gene classification and other phytoplasma genes as epidemiologic molecular markers. However the same disease is very often associated with molecularly differentiable phytoplasmas according with geographic distribution and different ecological situations (Table 1). On the other hand the inability to fulfil Koch's postulates severely restricts the understanding of the real roles of phytoplasmas in diseases and in plant-insect interaction.

Table 1. Main phytoplasma ribosomal group identified in grapevine worldwide.

Disease name	Phytoplasma 16Sr group-subgroup	'*Candidatus* Phytoplasma' or strain acronym	Country
Buckland valley grapevine yellows	16SrI	BVGY	Australia
Virginian grapevine yellows	16SrI-A	'*Ca*. P. asteris'	USA (Virginia); Canada
Grapevine aster yellows	16SrI-B	'*Ca*. P. asteris'	Italy; Portugal; Chile; South Africa; Canada; Turkey, Tunisia
Grapevine aster yellows	16SrI-C	'*Ca*. P. asteris'	Italy; Chile
Australian grapevine yellows	16SrII-A	'*Ca*. P. aurantifolia'	Australia
Virginian grapevine yellows	16SrIII-I	'*Ca*. P. pruni'	USA (Virginia)
Grapevine yellows	16SrIII-J	'*Ca*. P. pruni'	Chile
Grapevine yellows	16SrV-A	'*Ca*. P. ulmi'	Italy; Chile
"Flavescence dorée"	16SrV-C	FD-C	North Italy, France, Switzerland, Serbia, Slovenia, Croatia, Austria, Hungary
Palatinate grapevine yellows	16SrV-C	PGY	Germany
"Flavescence dorée"	16SrV-D	FD-D	North Italy; France, Spain, Portugal, Slovenia, Croatia
Chilean grapevine yellows	16SrVII-A	'*Ca*. P. fraxini'	Chile
Grapevine yellows	16SrX-B	'*Ca*. P. prunorum'	Italy, Hungary, Serbia
"Bois noir"	16SrXII-A	"Bois noir" (BN)	UE, Israel, Ukrain, Serbia, Bosnia & Erzegovina, Montenegro, Macedonia, Chile, Turkey, Canada, South Africa, Iran, China, Lebanon, Jordan, Canada, Georgia, Ukraine
Australian grapevine yellows	16SrXII-B	'*Ca*. P. australiense'	Australia

PHYTOPLASMA GRAPEVINE INTERACTION

The most studied diseases are "flavescence dorée" (FD) and "bois noir" (BN). FD is a quarantine disease transmitted by the leafhopper *Scaphoideus titanus*, subjected to mandatory control measures that over the last 30 years allow to reduce impact in affected grape growing areas. The major problem viticulturists are facing is the great ability of FD phytoplasmas to differentiate new strains in short periods of time that is of major relevance towards a correct disease management. Several studies were carried out in order to achieve genomic information related to the FD pathogenicity. A physical map of the 671 kbp chromosome was constructed and a map including the two rRNA operons, tuf, uvrB-degV and secY-map genes was produced (Malembic-Maher *et al*., 2008). This work was recently implemented by production of about 8300 FD-mapped reads assembled in 347 sequences, corresponding to 215 annotated genes. This allow to identify 10 unannotated genes, 15 polycistronic transcripts and three genes supposedly localized in the gaps of the FD92 draft genome. Functional classification revealed that the most expressed genes were either related to translation and protein

biosynthesis or hypothetical proteins with unknown function. Some of these latter were predicted to be secreted, acting as effectors with a potential role in modulating the interaction with the host plant. Interestingly, qRT-PCR validation of the RNA-Seq expression values confirmed that a group II intron represented the FD genomic region with the highest expression during grapevine infection. This mobile element may contribute to the genomic plasticity increasing its fitness towards host-adaptive strategies (Abbà *et al*., 2014). Post-translational protein modifications to study quantitative changes in the proteome and phosphoproteome of FD-affected and recovered grapevines, compared to healthy plants were determined. It was possible to identify 48 proteins that differentially changed in abundance and/or phosphorylation. Recovered plants were characterized by such changes for 17 proteins not detected in infected plants. Enzymes involved in the antioxidant response that were up-regulated in infected plants, such as isocitrate dehydrogenase and glutathione S-transferase, returned to healthy-state levels in recovered plants. Others belonging to the same functional category were down-regulated in recovered plants. Fifteen differentially phosphorylated proteins were identified in infected compared to healthy plants. Proteomic data were integrated into biological networks and their interactions were represented through a hypothetical model, showing the effects of protein modulation on primary metabolic ways and related secondary pathways (Margaria *et al*., 2013). Quantitative PCR was used to follow the seasonal changes of FD titre in grapevines from two vineyards located in climatically different vine-growing regions of Slovenia. In plants with high concentrations of FD in tissues with symptoms, phytoplasma was also detected in symptomless tissues. A trend of decreasing FD titre in all examined symptomless tissues from June to July and an increasing one throughout the growing season in symptomatic tissues was observed (Prezelj *et al*., 2012).

In almost all grapevine growing areas there is a wide presence of BN disease associated with phytoplasmas belonging to ribosomal subgroup 16SrXII-A recently classified as '*Candidatus* Phytoplasma solani' (Quaglino *et al*., 2013) that are transmitted to grapevine by *Hyalesthes obsoletus* Signoret (Homoptera, Cixiidae). Over the last fifteen years a severe spreading of BN disease was described in several European grapevine-growing areas and the usefulness of multilocus gene studies as epidemiologic marker was shown after the studies carried out on *tuf* gene (Langer and Maixner, 2004; Bertaccini *et al*., 2006; Mori *et al*., 2008). Molecular variability was found in several BN genes indicative for the presence of diverse strains, although it is still not clear how this is relevant to understand BN outbreaks (Cimerman *et al*., 2009; Pacifico *et al*., 2009; Fabre *et al*., 2011). At biological level the BN phytoplasma interaction with grapevine was studied demonstrating a progressive decrease of total chlorophyll, carotenoids, soluble proteins and ribulose-1,5-bisphosphate carboxylase activity in phytoplasma infected leaves (Bertamini *et al*., 2002). Effects of the BN infection on primary and secondary metabolic pathways were observed, including enzymes of the photosynthetic chain, Calvin cycle and lipid metabolism, the induction of defence genes and the suppression of cell wall degradation a set of genes whose expression patterns allowed the grouping of vines according to their infection status was identified (Albertazzi *et al*., 2009; Hren *et al*., 2009). The global transcriptional profiling in grapevine responses to BN was also studied in symptomatic and recovered plants: class III chitinase and phenylalanine ammonia-lyase and chalcone synthase expression were increased. In symptomatic leaves the expressions were up-regulated and also those of β -1,3-glucanase and flavanone 3-hydroxylase. For a moderately susceptible variety, the defense genes were generally up-regulated in both leaves from symptomatic and symptomless plants (Landi *et al*., 2011).

DETECTION

The phytoplasma detection by conventional and nested-PCR is a twenty years routine testing for grapevine, however rapid and accurate new tools were developed. A triplex real-time PCR assay detecting simultaneously FD and BN phytoplasmas with primers and probes specifically detecting the map gene of these two phytoplasmas was employed showing good intra-test and inter-test reproducibility (Pelletier *et al*., 2009). Multiple detection of FD and BN and of the viruses *Grapevine leafroll associated virus -1* and *-3* (*Ampelovirus*) and *Grapevine virus A* (*Vitivirus*) was achieved using the same crude extract as template. Quantitative reverse transcription for FD phytoplasma detection was found to be five orders of magnitude more sensitive than already used qPCR methods and it was successfully used to monitor infections in field and nursery samples (Margaria *et al*., 2009). Besides these approaches very recently a LAMP based assays was developed able to detect in 1 hour 16SrV group phytoplasma presence (Kogovsek *et al.,* 2015).

GEOGRAPHIC DISTRIBUTION

AFRICA. Aster yellows phytoplasmas were identified in Tunisian grapevine as belonging to16SrI-B subgroup strains (Mhirsi *et al*., 2004). In South Africa BN was detected in mixed infection with 16SrII phytoplasmas (Botti and Bertaccini, 2006a), and more recently a strong localized epidemic associated with a molecularly differentiable strain of aster yellows was reported (Engelbrecht *et al*., 2010; Carstens *et al*., 2011; Zambon *et al*., 2015) in which the insect vector *Mgenia fuscovaria* (Stål) was identified (Krüger *et al*., 2011).

AMERICA. In Canada BN phytoplasmas were identified in a grapevine plant of Grenache imported from Europe in 2006 (Rott *et al*., 2007) and in a survey in vineyards from British Columbia, Ontario and Québec phytoplasmas belonging to group 16SrI were detected in percentages reaching 6% in Ontario in 2008. Phytoplasma DNA was also detected in a number of potential insect vectors. DNA sequencing showed that phytoplasmas in insects and grapevines belong to 16SrI-A or 16SrI-B (Olivier *et al*., 2009). In USA grapevine yellows were mainly described in Virginia in highly susceptible varieties such as Chardonnay and more slowly in less susceptible varieties such as Cabernet Sauvignon (Davis *et al*., 1998; 2012). Several species of leafhoppers have been identified as potential vectors, including *S. titanus* (Beanland *et al*., 2006). In South America extensive studies were carried out in Chile where the symptomatic grapevine resulted to be infected by 16SrVII-A, 16SrI-B, -C, 16SrIII-J, 16SrXII-A and very recently also by 16SrV-A phytoplasmas. Experimental transmission trials indicate ability of *Paratanus* exitiosus (Beamer) to transmit 16SrIII-J phytoplasmas to grapevine (Bertaccini *et al*., 2006; Gajardo *et al*., 2009; Longone *et al*., 2011; Fiore *et al*., 2012; 2015a; 2015b).

ASIA. The presence of phytoplasmas in grapevine in Israel is know since aster yellows (16SrI) and western X (16SrIII) were reported (Orenstein *et al*., 2001), more recently also BN was identified (Zahavi *et al*., 2013). In 2009 Karimi *et al*. reported in the Korassan province of Iran phytoplasma strains related, but molecularly differentiable from BN, in symptomatic grapevine. More recently, in five provinces in the centre of Iran BN phytoplasmas were identified by RFLP and sequence analyses (Mirchenari *et al*., 2015). BN presence was also reported in Lebanon and Jordan (Choueiri *et al*., 2002; Salem *et al*., 2013). Phytoplasmas related to BN were identified in a little spot also in Shanxi province in China (Duduk *et al*., 2010) and together with 16SrVI phytoplasmas in Syria (Contaldo *et al*., 2011). A recent survey in Turkey on grapevines from Aegean, Central Anatolia and Western Anatolian regions allow to detect the presence of BN, and in some samples of 16SrI-B and 16SrIX phytoplasmas. The phytoplasma incidence rate was 18.33% mainly due to BN presence. Phytoplasma infections were present on 73.6% of wine grapevine cultivars and to a much less extent in table grapes (Canik *et al*., 2011; Ertunc *et al*., 2015).

AUSTRALIA. Grapevine diseases first molecular identification was of a phytoplasma related to BN and belonging to subgroup 16SrXII-B, named '*Ca.* P. australiense' (Padovan *et al*., 1995; Davis *et al*., 1997). Also 16SrII-A/-D subgroups and an aster yellows-related phytoplasma (16SrI) were identified (Gibb *et al*., 1999; Constable *et al*., 2003).

EUROPE. FD-associated phytoplasmas belong to ribosomal subgroups 16SrV-C and 16SrV-D and are further differentiated using polymorphisms in *rpS3*, *SecY* as well as other genes (Bertaccini *et al*., 1997; Angelini *et al*., 2001; Martini *et al*., 2002; Botti and Bertaccini, 2007; Arnauld *et al*., 2007). Both FD types resulted to be experimentally transmissible by the same vector *Schapoideus titanus* (Mori *et al*., 2002). Strains of FD 16SrV-D were detected in Northern Italy (Martini *et al*., 1999), France and Spain (Angelini *et al*., 2001; Torres *et al*., 2005) where the disease showed the highest epidemic outbreaks. In other grape producing areas such as North-central Italy and Serbia strains associated with disease outbreaks belong to ribosomal subgroup 16SrV-C (Marzachì *et al*., 2001; Duduk *et al*., 2004; Botti and Bertaccini, 2006b). In the recent years FD-D was identified in epidemic outbreaks in Amares region in northern Portugal. In the same region in 2002, similar but less severe symptoms were associated with 16SrI phytoplasmas, subgroup 16SrI-B (Sousa *et al*., 2010). In Austria (Reisenzein and Steffek, 2011) and in Switzerland (Jermini *et al*., 2014) the presence of FD is under strict monitoring, this is also valid for Hungary where it was officially reported in 2013 (EPPO, 2013). Very recent is the first record of FD in Germany in a nursery located in Rhineland-Palatinate (EPPO, 2014).

BN is found in all EU Countries growing grapevine. A survey of the grape growing area of Montenegro allow to detect BN phytoplasmas belonging to 16SrXII-A group tuf type-a and tuf type-b (Radonjicadonjic *et al*., 2009). A multiple gene analyses showed the presence of genetic variability among BN strains in diverse regions of the Republic of Macedonia associated with distinct ecologies (Kostadinovska *et al*., 2014). Recently in Austrian vine growing regions the multilocus analyses revealed a single genotype as predominant in stinging nettles and its 64% and 90% presence in grapevine and *H. obsoletus*, respectively. Interestingly, this genotype showed to be a tuf-b type with a different sequence named tuf-b2 (Aryan *et al*., 2014). BN phytoplasma presence was reported also from Ukraine (Milkus *et al*., 2005) and very recently from Georgia (Quaglino *et al*., 2014).

MANAGEMENT

The management of FD and BN was mostly studied and it is mainly based on reducing the presence of alternate hosts (plants and insects) in the affected vineyards, beside the uprooting of infected grapevine plants. While FD-C referable phytoplasmas were detected in alternate host plants such as *Clematis vitalba* and *Ailanthus altissima* (Angelini *et al*., 2004; Filippin *et al*., 2011) and are genetically related to alder phytoplasmas that are also believed to pay a role in strain differentiation (Arnaud *et al*., 2007), for the FD-D strains no alternative plant host or insect vector are reported. Recently new vectors or putative vectors for FD-C such as *Dictyophara europaea* (Filippin *et al.,* 2009) and *Orienthus ishidae* (Matsumura) (Mehle *et al*., 2010) were described. Another vector to grapevine is *Oncopsis alni* transmitting the Palatinate grapevine

yellows (16SrV-C phytoplasma molecularly differentiable from FD-C) in Germany (Maixner *et al*., 2000). BN phytoplasmas were reported to be vectored by *Reptalus panzeri (*Palermo *et al.*, 2004; Cvrković *et al*., 2014), *R. quinquecostatus* (Dufour) (Trivellone *et al*., 2005), *D. europaea* (Cvrkovic *et al*., 2011), *Neoaliturus fenestratus*, *Anaceratagallia ribauti* and *Macrosteles quadripunctulatus* (Riolo *et al*., 2006; Riedle-Bauer *et al*., 2008; Battle *et al*., 2008) although not always their transmission to grapevine was prouved. In Israel BN epidemiology is still unresolved since *H. obsoletus* is present but feeds mainly on *Vitex agnus castus* that was shown not to host the phytoplasma (Sharon *et al*., 2005; Dafny Yelin *et al*., 2015), although this plant species was recently found to host 16SrXII-A phytoplasmas in Montenegro (Kosovac *et al*., 2015).

Since the course of FD and BN diseases can result in either recovery or death of affected grapevines to establish whether there is an advantage in replacing symptomatic grapevines, data were collected on the costs of replacing in Chardonnay for BN and in Chardonnay, Merlot and Perera for FD. The costs of replacement decreased with the increase in the productive lifetime of the vineyards. The cost of maintenance was greatly influenced by the course of the diseases, and in the case of FD, also by the risk of new infections due to the presence of *S. titanus*. The replacement of plants affected by BN and FD is not profitable when recovery is the most frequent course of the disease, whereas it is necessary for cultivars where the course of the disease is frequently lethal (Pavan *et al*., 2012). Metagenomic DNA studies were carried out on healthy, phytoplasma diseased and recovered grapevine plants on endophytic bacterial community dynamic and diversity. When the plants are symptomless the endophytic bacterial community associated with diseased grapevines was different from those when the planta are symptomatic. The microbial community associated with recovered plants differs from that living inside healthy and diseased plants and the dynamic of bacteria previously reported as biocontrol agents such as *Burkholderia*, *Methylobacterium* and *Pantoea* was influenced by the phytoplasma infection process. Bacterial community composition is correlated to both phytoplasma infection and sampling date. In diseased plants, the pathogen infection process can decrease the impact of seasonality on bacterial community dynamic (Bulgari *et al*., 2011; 2014). Similar studies allow to verify the presence of bacterial endosymbionts that could modulate phytoplasma presence also in the FD and BN insect vector (Gonella *et al*., 2011). A study was performed to verify the efficiency of tissue culture techniques to eliminate BN by using stem cuttings and shoot tips associated or not to heat treatment, and stem cuttings combined with a hot water bath prior to culture initiation. The protocols were all suitable, either for shoot regeneration or for phytoplasma elimination. Stem cutting culture coupled with heat or hot water treatments appeared to be the most effective treatments leading to a good rate of survival and yielding up to 100% sanitized shoots (Chalak *et al*., 2013).

Recently it was demonstrated that phytoplasmas can grow in chemically defined media (Contaldo *et al*., 2012) and therefore deeper biological studies should be carried out to clarify the phytoplasma associated diseases epidemiology and management.

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OP 46 - Characterization of *vmp*1 gene of grapevine stolbur isolates from Bosnia and Herzegovina

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INTRODUCTION

In Bosnia and Herzegovina (B&H) '*Candidatus* phytoplasma solani' is molecularly identified in the vineyards of Herzegovina region as a causal agent of bois noir disease (Delić et al., 2006; 2011). In addition, molecular characterisation of tuf gene evidenced the presence of tuf-b type in tested stolbur grapevine isolates. Although *Hyalestes obsoletus* Signoret has determined to be the principal vector of stolbur phytoplasma of grapevine in Europe (Ayran et al., 2014), in B&H vineyards so far its presence was not confirmed. On the contrary, morphological identification of the collected insects from Herzegovina vineyards shows the presence of other *Auchenorrhyncha species* which were found to carry and transmit '*Ca.* phytoplasma solani' (Cvrković et al., 2011; 2014). However, *Reptalus quinquecostatus* (Dufour), and *Dictyophara europea* (Linnaeus), *were* found to be dominant in surveyed vineyards (Delić et al., 2011; 2013). Generally speaking there are lot of gaps in bois noir epidemiology in B&H. Gene encoding putative stolbur phytoplasma membrane protein (*vmp*1) showed to be useful in studies of phytoplasma-insect vector interactions (Fabre et al., 2011). Therefore, stolbur isolates from infected grapevine were selected for molecular characterisation of the *vmp*1 gene in order to get better view of '*Ca.*phytoplasma solani' *vmp*1 strains presence and to obtain more information of their epidemiological cycle.

MATERIALS AND METHODS

Eighteen '*Ca.*phytoplasma solani' isolates previously identified and characterised as a tuf-b type were selected for further *vmp*1 gene strain characterisation. All samples were collected from Herzegovina region, the main viticulture center, vineyards (Table1). Partial vmp1 gene was amplified in a nested-PCR assay using StolH10F1/StolH10R1 primers (Cimerman et al., 2009) followed by TYPH10F/TYPH10R (Fialová *et al*., 2009) primer pair. The Obtained nested-PCR products were digested with *Rsa*I enzyme and were separated by electrophoresis in 2% MetaPhor agarose (Cambrex) gel stained in ethidium bromide. Specific *vmp*1 restriction pattern representatives were chosen for sequencing and phylogenetic analyses. TYPH10F/R nested PCR products were sequenced in both directions using Sanger method on Macrogen3730XL05-16108-002 instrument. Multiple sequence alignment was conducted in muscle v3.8.31 (Edgar, 2004) and subsequent phylogenetic analysis was performed using MrBayes 3.2 (Ronquist et al., 2012).

RESULTS AND DISCUSSION

Amplicons of *vmp*1 gene of about 1450 bp were successfully obtained from all samples. Restriction analyses with *Rsa*I of obtained fragments showed the presence five different profiles, namely V9, V14 and V4 and the combination of V14+V9 and V4+V9 (Figure 1;Table 2). The most frequent profile was V9, identified in nine out of eighteen stolbur grapevine isolates from all surveyed locations, followed by V14 and V4. Phylogenetic analyses confirmed the presence of the selected amplicons in the same clades with reference sequences having the same pattern. Moreover phylogeny indicated that our isolates may have the same origin with Italian ones. Nevertheless, our results showed a particular genetic variability with the dominance of V9 pattern which was not previously found to be very abundant (Murolo et al., 2010; 2013). All in all these results gave a good background to better understand and clarify BN disease epidemiology in B&H.

Table 1. List of the samples used in the study RFLP and sequencing results and corresponding GenBank accession numbers.

Stolbur grapevine isolates	Location	*vmp*1 profile[a]	*vmp*1 GenBank acc. no.
5/09	Trebinje	V9	-
20/09	Trebinje	V9	-
30/09	Trebinje	V14	KP739860
154/10	Popovo polje	V14+V9	-
155/10	Popovo polje	V9	-
3/11	Ljubuški	V9	-
11/11	Popovo polje	V14+V9	KP739859
12/11	Popovo polje	V14	-
14/11	Popovo polje	V14	KP739857
17/11	Popovo polje	V4	KP739858
55/11	Mostar	V9	-
66/11	Ljubuški	V9	-
G-4/13	Ljubuški	V9	-
G-6/13	Ljubuški	V9	-
G-21/13	Ljubuški	V4+V9	KP739861
G-22/13	Ljubuški	V9	-
G-23/13	Ljubuški	V9	-
G-24/13	Ljubuški	V9	-

[a]vmp1 RFLP profile profiles according to Murolo et al. 2010;2013.

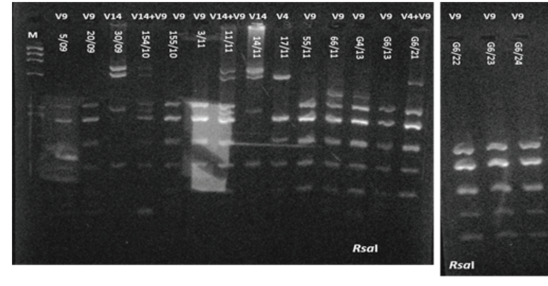

Figure 1. *Rsa*I restriction profiles of TYPH10F/R PCR products; **M:** *ΦX174 DNA*-Hae III DNA marker (Fermentas, Lithuania).

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OP 47 - Cultivation in chemically defined media of phytoplasmas from field-infected grapevine plants showing yellows symptoms

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INTRODUCTION

Phytoplasma cultivation was achieved using as a source micropropagated phytoplasma infected periwinkle shoots (Contaldo *et al*., 2012; 2013). In order to verify the possibility of cultivation from field infected samples, phytoplasmas associated with grapevine yellows were employed. “Flavescence dorée” (FD) phytoplasmas belonging to ribosomal subgroups 16SrV-C and 16SrV-D (Martini *et al*., 1999) and “bois noir” (BN) phytoplasmas belonging to ribosomal subgroup 16SrXII-A were selected for these trials. Since both diseases are spread by sap feeding hemipteran insect vectors, and by exchanges of infected planting material from grapevine nurseries, the growth of these phytoplasmas in chemically defined media would facilitate the study of their biology and pathogenic mechanisms.

MATERIALS AND METHODS

Plant material. Canes from symptomatic and asymptomatic grapevine plants were collected in 15 year-old vineyards of cv Glera located in Treviso province (Italy). Phytoplasma identification was carried out by nested-PCR amplification using P1/P7 followed by M1/B6 (=758F/B6)(Gibb *et al*., 1995; Padovan *et al*., 1995) primer pairs under amplification conditions described by Schaff *et al*. (1992). RFLP analyses were then carried out on amplicons with specific informative enzymes.

Phytoplasma isolation. From each sample two midribs were surface sterilized, dried, moistened in PivL (Contaldo *et al*., 2012), sliced and incubated at 25±1°C. Uninoculated tubes were also maintained under the same conditions. The tubes were inspected for signs of a colour change from orange-red (pH above 7.0) to yellow (pH below 6.8). When acid colour changes occurred broth cultures were inoculated onto TSB-agar plates (Oxoid, UK) and incubated under previously described conditions (Contaldo *et al*., 2012).

The agar surface was observed with optical bifocal microscope at 40X magnification. Distinctive single colonies were then picked and transferred into fresh liquid medium, for purification following a slightly modified published procedure (Anonymous, 1979). Single colonies were collected separately, dissolved in distilled deionized sterile water (DDSW) and subjected to nucleic acid extraction by DNeasy Plant Minikit (Qiagen, Germany). At the same time nucleic acid was also extracted from the corresponding tubes and from uninoculated broth as negative control, after alkaline lysis and a phenol/chloroform based procedure (Pourbakhsh *et al*., 2010).

Phytoplasma identification from cultures was carried out by specific nested-PCR assays on 16S rDNA gene followed by RFLP analyses with *Tru*1I and *Taq*I. Direct sequencing of selected PCR products was performed using R16(I)F1/R1 (Lee *et al*., 1994) and M1/B6 primers.

RESULTS AND DISCUSSION

Among the phytoplasma positive grapevine identified after PCR/RFLP analyses the three plants employed for phytoplasma cultivation were Yan, infected with BN and 05M and 7Bariv, infected respectively with FD-C and FD-D phytoplasmas. In the liquid medium inoculated with Yan sample, the acidification appeared after 7-10 days from isolation, while in tubes inoculated with 05M and 7bariv samples colour change was observed after 36/48 hours. Uninoculated tubes did not show colour changes, while tubes inoculated with healthy grapevine midribs in some cases gave rapid acid colour changes possibly caused by plant endosymbionts (Hardoim *et al*., 2008). The BN-Yan isolate acidification time was shorter than the one of CH-1 strain of BN (Contaldo *et al*., 2012) even if they both are assigned to the same ‘*Candidatus* Phytoplasma solani’ species (Quaglino *et al*., 2013). This could be explained considering that the CH-1 strain was isolated from periwinkle shoots maintained under micropropagation for longtime since it is reported for some phytoplasmas the loss of biological characteristics such as insect transmissibility (Denes and Sinha, 1992), and plasmid related with insect transmissibility (Nishigawa *et al*., 2002) under these conditions.

In agar medium small colonies were observed after 48-72 hours and colonies visible by naked eye after 4 days from all the isolated strains (Fig. 1). Nested-PCR assays confirmed the phytoplasma presence and identity in both DNA from

those colonies and corresponding broth sample tested; no amplification was observed from negative controls. The BN-Yan isolate RFLP profile was identical to the one of the CH-1 reference strain and to the BN strain amplified from field-collected sample Yan, employed for isolation. The RFLP profiles obtained from cultures of FD-05M and FD-7bariv showed that the *Taq*I profiles were referable to FD-C and FD-D respectively, and were identical to those of the original strains from grapevine tissues.

Direct sequencing of a R16(I)F1/R1 amplicon from BN-Yan colonies produced a fragment that was 100% identical to sequences of the majority of "stolbur" strains available in Genbank and to the sequence of the original strain employed for isolation. The direct sequencing of M1/B6 amplicon from colonies obtained from strains FD-05M and FD-7bariv produced fragments showing 99% homology among each others and with FD, elm yellows and alder yellows strains available in Genbank, as well as to the sequences of the original strains employed for isolation. In the DNA sequence from cultivated a FD strains there is one SNP in position 4 in FD-05M to the original strain from grapevine and to the Genbank reference strain HQ712064. Moreover one SNP in position 234 is present in the cultivated strain FD-7bariv to the original strain from grapevine and to the Genbank reference strain AJ548787. The 100% identity to BN strains of the 16S rDNA sequence from cultivated BN-Yan is in agreement with previous results obtained on cultivated CH-1 strain in periwinkle (Contaldo *et al*., 2012). The presence of 99% homology on 16S rDNA between FD-C/-D DNAs from colonies and their respective original grapevine samples, was reported for one of the cultivated strains belonging to ribosomal group 16SrII. However considering that the SNPs detected are not restriction sites, and are therefore nonsynonimous substitution, their presence is not affecting the phytoplasma classification.

This is the first successful cultivation of phytoplasmas directly from naturally infected plants host, and also of phytoplasmas belonging to subgroup 16SrV, in particular of the agents of the "flavescence dorée" disease of grapevine.

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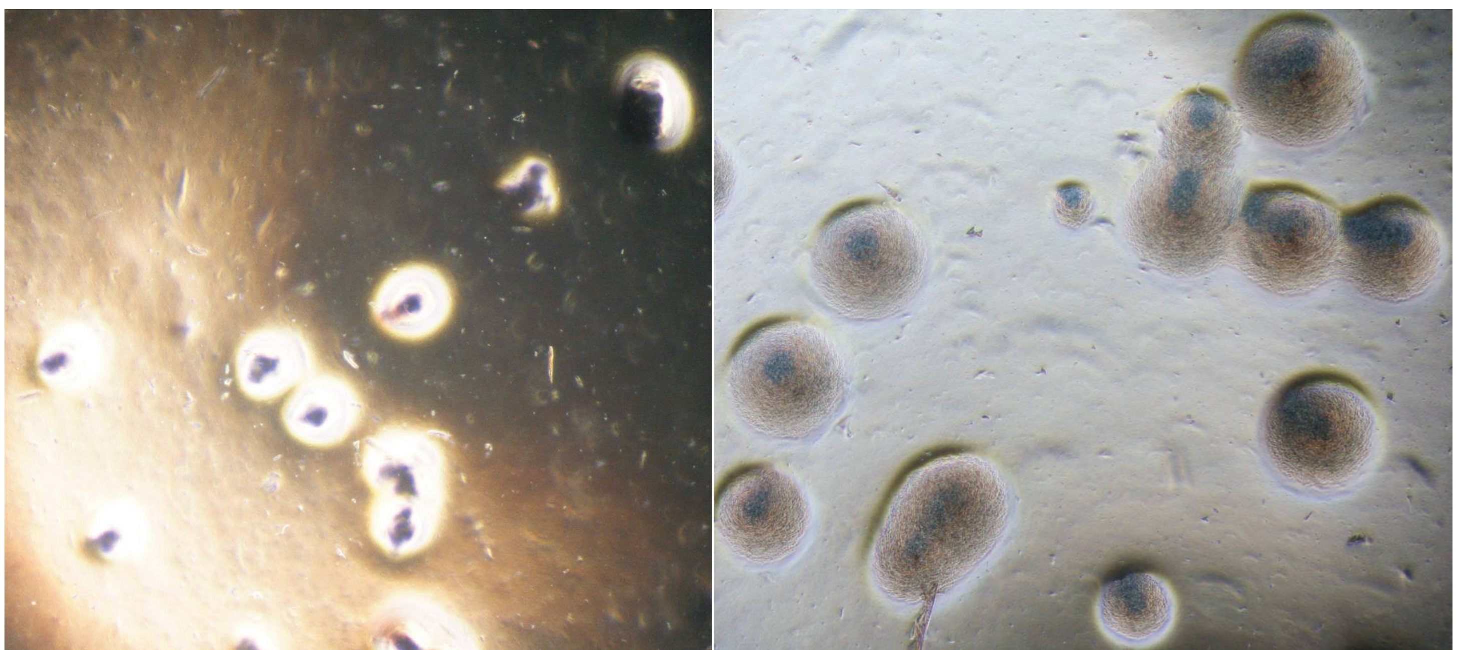

Phytoplasma colonies from infected grapevine photographed after 4 days incubation: left FD-7bariv and right BN-Yan (Bars = 250 µm).

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OP 48 - ‘Bois Noir’ phytoplasma disease in grapevine in Azerbaijan

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INTRODUCTION

‘Bois noir’ (BN) and ‘Flavescence dorée’ (FD) are the two most important diseases of grapevine caused by phytoplasmas. BN disease, caused by ‘Candidatus Phytoplasma solani’, is widespread in all European and Mediterranean viticultural areas, and it can seriously affect quality and quantity of production. Up to now there were no reports about grapevine phytoplasma diseases in Azerbaijan,. Recently, grapevines showing symptoms suggesting phytoplasma infection have been observed in several grape-growing areas in Azerbaijan. During surveys conducted to main grape-growing areas in Azerbaijan, the white varieties of grapevine with the leaf rolling and yellowing and red varieties with the leaf reddening symptoms were collected in September 2014. The aim of this study is to test the hypothesis of a phytoplasma aetiology.

MATERIALS AND METHODS

Total DNAs were extracted from 1g fresh leaf midribs of diseased and symptomless plants (as control) following classical CTAB extraction protocol. The DNA concentrations were measured by a nanospectrophotometer. Total nucleic acid extracts were tested by 16S-rDNA nested PCR with universal primer pairs R16mF2 / R16mR1 and R16F2n / R16R2 (Gundersen and Lee, 1996). 16S Nested PCR products were submitted to RFLP analyses with enzymes *Alu*I and *Taq*I. Phytoplasma strains FD-70 (Flavesence doree phytoplasma, 16SrV-C), Stolbur-Moliere (‘Ca. P. solani’, 16SrXIIA) maintained at INRA Bordeaux in *Catharanthus roseus* were used as references. Digested PCR products were analyzed by 3% agarose gel electrophoresis and visualized by staining with ethidium bromide under UV. Stolbur group specific nested-PCR test based on *stamp* gene amplification (Fabre et al. 2011) were performed using the primer pairs Stamp-F/R0 and Stamp-F1/R1.

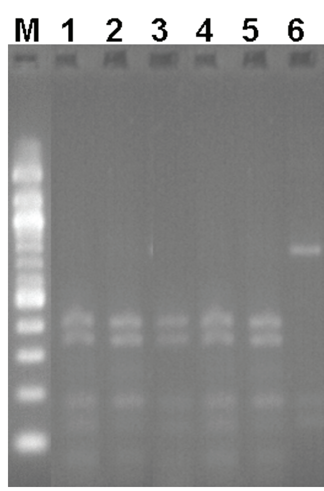
RESULTS AND DISCUSSION

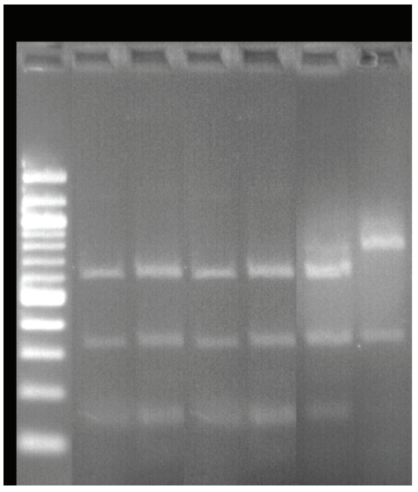
Total DNA from twenty eight grapevine leaf samples with the leaf rolling and yellowing for white varietes and red varieties with the leaf reddening symptoms also from two symptomless plants collected in September 2014 were extracted and obtained DNA extracts were tested by 16S-rDNA Nested PCR. While symptomless plants gave no amplification, positive results (1250 bp amplicons) were obtained from four samples of red variety of grapevine samples collected in Absheron.

Table 1. Grapevine samples collected from Ganja, Guba and Absheron regions.

Sample ID	Collected region	+ nested PCR 16S R16R2-F2n /number tested
GR1.AZ to GR15.AZ	Ganja	**0/15**
GR16.AZ to GR20.AZ	Guba	**0/5**
GR21.AZ to GR28.AZ	Absheron	**4/8**

For identification of detected grapevine phytoplasmas, all of the 16S-rDNA Nested PCR products obtained were subjected to overnight restriction with enzymes *Alu*I and *Taq*I. As shown from the Figure 1, 16S-rDNA-RFLP patterns of *Alu*I and *Taq*I from all of the four grapevine samples gave the same profile that the Stolbur-Moliere ‘*Ca.* P. solani’ reference isolate.





***Alu*I** ***Taq*I**

Figure 1. 16S-rDNA Nested PCR-RFLP analysis.

M-100 bp marker (Sigma), 1-GR 21.AZ; 2-GR 21.AZ;

3-GR 21.AZ; 4-GR 21.AZ; 5-St MOLIERE; 5-FD-70

All positive grapevine samples were tested by '*Ca.* P. solani' specific nested-PCR based on *stamp* gene amplification. Nested PCR products were obtained for the four grapevine samples previously positives for 16S phytoplasma amplification. 'Ca. P. solani' was therefore recognized as the causal agent of grapevine diseases showing leaf reddening in Absheron region of Azerbaijan '*Ca.* P. solani' has recently been detected in Azerbaijan in annual crops such as eggplant, pepper and tomatoes, but also in declining cherry and common meddlar trees (Balakishiyeva et., 2010). *Hyalesthes obsoletus*, and a '*Ca.* P. solani'-infected cixiid planthopper initially reported as *Setapius sp.* but finally as assigned to *Hyalesthes noahi* are known to be present in Azerbaijan (Balakishiyeva *et al.,* 2013).

It is the first report of grapevine phytoplasma diseases and the first report of '*Ca.* P. solani' associated with grapevine in Azerbaijan.

ACKNOWLEDGEMENTS

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OP 49 - Epidemiology of aster yellows phytoplasma: alternate host plants and the vector *Mgenia fuscovaria* (Hemiptera: Cicadellidae) in South Africa

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INTRODUCTION

Aster yellows phytoplasma (AY), caused by '*Candidatus* Phytoplasma asteris' (16SrI-B group), is a disease of grapevine (*Vitis vinifera* L., Vitaceae) of phytosanitary concern in South Africa (Engelbrecht et al., 2010). It is transmitted to grapevine by the indigenous leafhopper *Mgenia fuscovaria* (Stål) (Hemiptera: Cicadellidae) (Krüger et al., 2011). AY is restricted to grapevine-growing regions in the Western Cape. It has a wide host range, infecting fruit and vegetable crops as well as wild plants (Hogenhout et al., 2008). However, little is known about the epidemiological role of alternate host plants, which may constitute important reservoirs and sources of inoculum of AY in vineyards. The aim of this study was to identify alternate host plants of AY naturally occurring in vineyards, which may serve as a local inoculum source, and in controlled transmission experiments with *M. fuscovaria*.

MATERIALS AND METHODS

Wild and crop plants were sampled in and around vineyards infected with AY in the Vredendal region in the Western Cape between 2009 and 2014. Leaf material collected from plants was stored at -70 °C until analysis with real-time PCR. DNA was extracted from plants using the NucleoSpin Plant II kit (Macherey Nagel). Real-time PCR was performed using the LightCycler® TaqMan® Master kit. The LightCycler technology and the protocol were adopted from Angelini et al. (2007).

M. fuscovaria and other leafhoppers were monitored weekly in an AY-infected vineyard since 2009 using 10 yellow sticky traps per week. In addition, leafhoppers were sampled in different seasons with sweep netting and plants were inspected for leafhoppers.

In order to determine whether *M. fuscovaria* is able to transmit AY to host plants other than grapevine, controlled transmission experiments were carried out with field-collected adults. Plants were grown from seed at the University of Pretoria (Gauteng). Transmission experiments were carried out in Vredendal (Western Cape). Depending on the number of leafhoppers available between 1 and 25 individuals were exposed to single plants for an inoculation access period (IAP) of at least 24 hours.

Adults used in transmission experiments and subsamples of field-collected *M. fuscovaria* were preserved in 95% ethanol. DNA was extracted from single leafhoppers using a 2% CTAB extraction method or an unpublished DNA buffer extraction method developed by Jean Peccoud and Nicolas Sauvion (French National Institute for Agricultural Research (INRA)) for psyllids. Samples were tested for the presence of AY with real-time PCR following Angelini et al. (2007).

RESULTS AND DISCUSSION

Of the 1129 field-collected plants belonging to 14 families, 28 genera and 34 species, 11 species tested positive for AY: Apocynaceae: periwinkle (*Catharanthus roseus* (L.) G. Don; Asteraceae: blackjack (*Bidens bipinnata* L.), white goosefoot (*Erigeron bonariensis L.*), sow thistle (*Sonchus oleraceus* L.); Brassicaceae: wild radish (*Raphanus raphanistrum* L.); Cucurbitaceae: *Cucurbita* sp.; Poaceae: sticky bristle grass (*Setaria verticillata* (L.) P. Beauv.), triticale (*Triticosecale* sp.), maize (*Zea mays* L.); Urticaceae: small stinging nettle (*Urtica urens* L.). In controlled transmission experiments with five plant species and *M. fuscovaria* as vector, none of the 32 periwinkle plants and none of the 8 blackjack plants exposed to adult *M. fuscovaria* tested positive for AY, although 24% of 262 leafhoppers tested were positive for the phytoplasma. *M. fuscovaria* successfully transmitted AY to maize and triticale, as well as wheat (*Triticum aestivum* L., Poaceae), which did not form part of the field collected plant samples. Triticale is planted as a cover crop in vineyards. The results show that *M. fuscovaria* is able to transmit AY to some Poaceae as well as grapevine.

Adults and nymphs of *M. fuscovaria* are present in vineyards throughout the year. Adults were observed feeding on a number of herbaceous hosts. Initial seasonal trends show that the number of AY-infected adults was higher in autumn (43%) than in summer (20%) or winter (12%). Numbers of adult *M. fuscovaria* are usually high during the winter months and alternate hosts such as triticale may serve as an important source of inoculum in vineyards. However, before devising management strategies, it is important to determine whether AY can be transmitted by *M. fuscovaria* from grapevine to alternate hosts and *vice versa*.

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OP 50 - The genetic variability of AY in South African vineyards and its spatial and temporal distribution in individual vines

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INTRODUCTION

Grapevine yellows (GY) is an emerging threat to the South African grapevine industry and efforts are underway to prevent further spread of this devastating disease. Detection of Aster yellows, the main agent associated with GY in South Africa, is notoriously erratic. In order to better understand the genetic variability of this pathogen, PCR and RFLP analysis of ribosomal (16S rRNA) and house-keeping genes (*amp*, *groEL*, *rp* and *secY*) were performed on AY-infected vines from different geographical regions in South Africa. Moreover, to improve AY detection, the spatial and temporal distribution of AY in infected vines was monitored over two growing seasons.

MATERIALS AND METHODS

For the PCR and RFLP analysis, leaf samples were collected from 28 symptomatic grapevine plants in three vineyards in Vredendal and one in Robertson, South Africa. Total DNA was extracted using both a commercial kit (Macherey-Nagel, Düren, Germany) and a chloroform/phenol based extraction protocol (Prince *et al*., 1993). Nested-PCR assays were carried out for the genes 16S *rRNA*, *groEL*, *amp* and *secY*, as described by Zambon *et al*., 2015. PCR products were digested using the following restriction enzymes: *Hha*I and *Tru1*I for the 16S *rDNA*; *Tru1*I and *Alu*I for the *rp, amp, groEL, secY* genes. The resulting RFLP profiles were then compared to those obtained from two Italian AY strains (FD-T and REPT) and also reference strains maintained in a periwinkle collection: NJ-AY and GDI (16SrI-A), MBS and AY-J (16SrI-B), AVUT (16SrI-B/M), KVF and KVG (16SrI-C), ACLR-AY (16SrI-F). Selected amplicons obtained from the different genes were sequenced in both directions. Sequences were assembled using CLC Genomics Workbench 7 (http://www.clcbio.com) at default parameters and then aligned using Clustal X (Thompson *et al*., 1997).

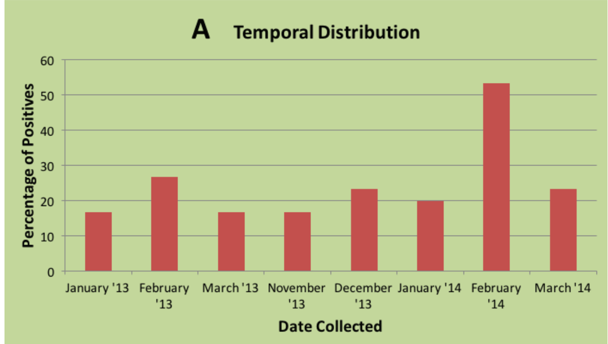
The *in planta* spatial and temporal distribution of AY was determined in a severely infected Chardonnay vineyard in Vredendal. DNA for temporal distribution studies was extracted from cane scrapings (phloem tissue) of 30 individual plants using a NucleoSpin II Plant DNA Extraction Kit. Samples were collected at monthly intervals over two growing seasons. DNA for the spatial distribution study was extracted from five tissue types (root, trunk, cane, petiole and leaf) of three whole Chardonnay plants (plants 2, 4 and 7) from the same vineyard. A triple nested PCR assay using universal primers (Lee *et al*.,1994) was used for AY detection in all these samples. Selected amplicons obtained from the different assays were sequenced in both directions to verify the identity of AY.

RESULTS AND DISCUSSION

Aster yellows-related strains (16SrI-B) were identified in all the symptomatic grapevines collected in the different vineyards. After its identification and characterization using the 16S rRNA gene, 15 samples were selected for further multigene analyses. Amplicons of the *rp* gene were successfully generated from all samples and restriction analysis with *Tru1*I and *Alu*I enzymes showed that all samples but one (S15) had identical profiles, albeit different from those available in literature. In the case of *groEL* gene typing, *Tru1*I and *Alu*I restriction analysis yielded fragments that were identical among all samples, and also identical to that of reference strain AY-J (16SrI-B). The RFLP analyses carried out on *amp* amplicons showed some variability among the South African grapevine samples, including the positive control APW (from periwinkle), however five samples did not amplify. Finally, the RFLP analysis carried out on the gene *secY*, showed the presence of four distinct groups. Samples 1Y, 2Y, S4 and APW were identical in their restriction patterns; a different profile was observed for samples S13, S14, S17, while samples Robertson 1 and Robertson 2 showed a third profile type. A unique profile was also observed in sample S15. The *secY* gene showed greater variability and four groups could be delineated, none of which matched any reported profile. In order to confirm the results obtained by the RFLP analysis, selected amplicons were sequenced. Sequence data confirmed the differences in some of the analyzed genes, demonstrating the considerable variability present in the *secY* gene.

Temporal distribution patterns showed a distinct peak in the late summer (February) for both of the years of monitoring (Fig.

1A). The spatial distribution in individual plants was not consistent between the three plants tested. Taken together however, the most positive AY detections in these plants were from phloem scrapings of cane or trunk tissues (Fig. 1B). Interestingly, petiole tissue seem to be rather unreliable as a source for AY detection.



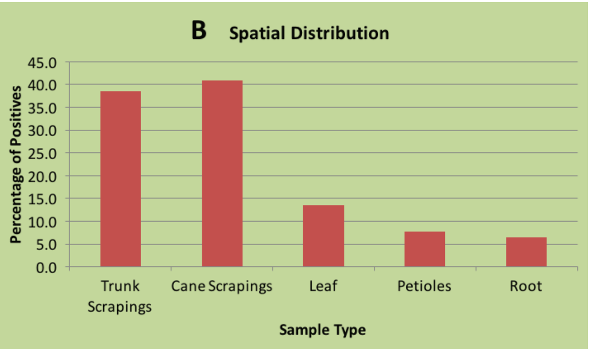


Figure 1. Temporal (A) and spatial (B) distribution of AY in diseased grapevine plants.

Our results showed a low level of genetic variability among AY isolates detected in South African vineyards, which may suggest a recent, single introduction of this pathogen into local vineyards. Of the genes evaluated, only the *secY* gene seems to be useful as an informative genetic marker since, for the first time, it made possible the differentiation of aster yellows isolates infecting grapevines in two different regions of South Africa. The spatial and temporal results confirm earlier reports on the unreliability of AY detection in grapevine. Our results suggested that the optimal detection regime comprise testing of phloem scrapings of cane tissue during the late summer, just before harvesting. Studies like this contributes to a better understanding of this economically important pathogen in order to develop effective management practises for Grapevine yellows in the region.

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OP 51 - Grapevine phytoplasma infections in Turkey

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INTRODUCTION

Turkey is located in the subtropic climatic region of theworld and its Northeastern part, the Anatolian peninsula, located between Black sea and Caspian sea regions, includes the location from which many important grapevine varieties originate. In Turkey, grapevine cultivation is present since more than 6000 years, and there is veryrich potential of both wild (*Vitis vinifera ssp. sylvestris*) and cultivated grapevine *(Vitis vinifera ssp. sativa*) germplasms. According to FAO data, Turkey has 540,000 ha of grapevine cultivated area, ranking fourth after Spain, France and Italy, and a grapevine production of 3,923 million tons, ranking sixth after Italy, France, China, USA and Spain (Soylemezoglu et al. 2015). Viticulture provides 24.8% of the total yearly fruit production andcomes after field crops, fruit and vegetable production (TUIK, 2008). The main viticultural region is the Aegean Region with 151,401 ha of vineyard area, that is the 31% of the grapevine cultivated area of the country, followed by Mediterranean, Central Anatolia, Eastern Anatolia, and Marmara regions. Worldwide several phytoplasmas, were associated

with diseases of grapevine such as, aster yellows (group16SrI), elm yellows (group 16SrV) and “stolbur” (subgroup16SrXII-A), together with phytoplasmas belonging to 16SrII, 16SrIII, 16SrVII, and 16SrX groups (Angelini 2010; Constable 2010; Duduk et al. 2010). Recently, severe redness and inward curling of the leaves were observed in wine vineyards in Turkey,therefore intensive surveys were conducted in the main viticultural production areas to verify phytoplasma presence and identity.

MATERIALS AND METHODS

Phytoplasma survey

Periodical surveys were conducted in main viticultural areas located in Thrace (Edirne, Kirklareli, Tekirdag),Aegean region (Izmir, Manisa and Denizli), CentralAnatolia (Ankara, Nevsehir) and Eastern Anatolia region(Elazig, Malatya, Diyarbakir and Mardin) ofAnatolian Peninsula from July-September in 2009-2010. Leaf and young shoot samples were collected from 289 symptomatic and 20 non-symptomatic plants.

Nucleic acid extraction

Nucleic acids were extracted from 1 g of grapevine leafribs, using a chloroform/phenol protocol described in Prince et al. (1993), suspended in 50-100 µl of TEbuffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) and stored at -20°C until they were processed. DNA’s of non-symptomatic plants were used as negative control.

PCR and nested PCR assays

Direct PCR assays with ribosomal P1/P7 universal primer pair (Deng and Huruki 1991; Schneider et al. 1995), followed by nested-PCR with R16F2n/R2 (Gundersen and Lee 1996) or M1/B6

(Duduk et al. 2004) were carried out. R16F2n/R2 amplicons were used as templates in a second nested PCR with R16(I) F1/R1, R16(V)F1/R1 (Lee et al. 1994) and M1/M2 (16R758f/16S1232r) primer pairs (Gibbs et al. 1995).

RFLP analyses

Preliminary identification of the detected phytoplasmas was achieved by RFLP analysis of R16(I)F1/R1,M1/B6 and M1/M2 amplicons with restriction endonucleases *Tru1*I and *Taq*I.

RESULTS AND DISCUSSION

Survey results

During the surveys, 159 and 130 symptomatic grapevinesamples were collected in the main viticultural areas of Turkey in 2009 and 2010, respectively. Mainsymptoms observed were severe redness and inwardcurling of the mature leaves (Fig. 2). Other typicalyellows symptoms such as leaf yellowing and triangle shaped, were not detected. Symptoms were usually present on the old mature leaves, occasionally, in some grapevine varieties, the whole canopy was completely red. The redness of the leaves was more severe and frequent on winegrapevine varieties comparing to table grapevines.

PCR and nested PCR

After double nested-PCR assays with primers M1/M2,53 out of the 289 grapevine sample tested were detected as phytoplasma-infected. The detection rate of phytoplasma-infected plant samples was 18.33%; in 49 samples ‘Ca. P. solani’, was identified according to the restriction profiles indicating the presence of phytoplasmas belonging to 16SrXII-A.Positive samples were also amplified in second nestedPCR assays with primers R16(V)F1/R1 and R16(I)F1/R1. In two samples, phytoplasmas belonging to 16SrV group were detected, while all other samples were amplified with R16(I)F1/R1 primers and after RFLP analyses, 16SrI-B and 16SrXII-A phytoplasmas were detected respectively in 1 and 49 samples. One sample showing a profile not referable to any one reported in literature was subjected to sequencing and the 1,063 bp sequence (accession number HQ714331), showed 99% homology with phytoplasmas belonging to group 16SrIX-C. M1/M2 (about 440 nt) amplicons of the BN strains showed 100% homology with sequences of phytoplasmas belonging to the same group present in the NCBI Genebank (accession numbers KJ810633-KJ810649). Sequence analyses of two samples (K11 KP015027 and C24 KP015028) amplified with M1/B6 primers (843 and 845 nt respectively) showed 99% homology with ‘Ca. P. solani’-related strains, while the RFLP analyses with TaqI showed the presence of an extra band of 700 bp in strain K11 that was not referable to other reported phytoplasmas nor retrivable by in silico RFLP. The incidence of grapevine phytoplasmas in Turkish vineyards was low and the predominant phytoplasma, infection was associated to the presence of “bois noir” phytoplasma.*Hyalestes obselatus* was not so common in the vineyards where as *Scaphoideus titanus* was not detected in Turkey.

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OP 52 - Results from the 'Epidemiological studies on reservoir hosts and potential vectors of grapevine flavescence dorée and validation of different diagnostic procedures" Project

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INTRODUCTION

Grapevine "flavescence dorée" (FD) is a severe grapevine disease of high economic impact for vine growers, nurseries and provincial governments in all European grape growing areas and the associated organism is of quarantine concern in Europe (directive 2000/29 EC). Genetic analysis of FD genome with different molecular markers revealed a population variability and the presence of different FD strains in the 16S rDNA, belonging to subgroups 16SrV-C and 16SrV-D (Martini *et al*., 1999; Arnaud *et al*., 2007). The main hosts for this phytoplasma are *Vitis vinifera* and *V. riparia*, while the most important vector is the well-known leafhopper *Scaphoideus titanus* Ball. However, interest has been focused on several wild host plants, found infected by FD e.g. *Clematis vitalba*, *Alnus glutinosa* (Malembic-Maher et al., 2009), *Ailanthus altissima* (Filippin *et al*., 2010) and on some other leafhoppers able to harbor FD phytoplasma e.g. *Dictyophara europaea* (L.) (Filippin *et al*., 2009) and *Orienthus ishidae* Matsumura (Gaffuri *et al*., 2011; Mehle *et al*., 2011). The laboratory detection of FD phytoplasma is difficult mainly due to its irregular distribution in the plant tissues, low phytoplasma titer in latently infected plants, seasonal variability in phytoplasma concentration and the presence of other phytoplasmas inducing similar symptoms. The EUPHRESCO GRAFDEPI Project was aimed: i) to increase the knowledge about FD disease epidemiology, with special focus on the role of reservoir hosts of the phytoplasma alternative to grapevine and on presence of other potential insect vectors beside the reported *S. titanus*; ii) to harmonize the phytoplasma detection protocols within the EU by performing inter-laboratory comparison of the most common diagnostic procedures to verify their reliability towards validation parameters; iii) to provide elements for the development of a more efficient control of the disease.

MATERIALS AND METHODS

The Project, involving 15 Institutions (Table 1), was organized in three scientific Work Packages, focused on: i) epidemiological studies; ii) validation of diagnostic procedures; iii) design of surveillance systems.

Epidemiological studies. To generate testable hypothesis on transmission mechanisms and dynamics of FD the presence of wild plants as reservoir and new potential insect vectors were monitored in vineyards and in surroundings in different regions. Moreover, case investigations were done by FD strain characterization of selected samples to get an overview on the prevalence and distribution strains. The *16S rRNA* and *secY* genes were used to detect FD strains and get insights into their molecular variability. Finally, data on the susceptibility of local and international varieties based on visual inspections and on an evaluation scheme were collected.

Validation of diagnostic procedures. Seven molecular protocols were compared in inter-laboratory trials. The tested protocols consisted of universal and group-specific real-time and conventional nested PCR assays. For each protocol, an identical series of 24 blind target (11) and no-target (13) samples was analyzed to obtain data for the calculation of performance criteria, according to UNI CEI EN ISO/IEC 17025: i) diagnostic sensitivity (SE); ii) diagnostic specificity (SP); iii) last level at 100% positive results; iv) reproducibility; v) repeatability.

Design of surveillance systems. The activity was mainly based on the results on new knowledge on alternative FD control strategies. Data obtained has been employed to establish the risk connected with new phytoplasma reservoir plants and possible insect vectors in spreading of the disease, whereas data from WP3 to individuate suitable analytic tests to be used in different monitoring situation (commercial orchards, nurseries, mother plant fields, symptomatic and asymptomatic samples). On the basis of these data, surveillance schemes were outlined with the aim to harmonize the disease containment measures within the EU.

RESULTS AND DISCUSSION

Epidemiology. The possibility to share cases and outbreak studies has allowed to define some hypothesis updating the epidemiologic cycle of FD disease. Among wild plants tested for FD, only *C. vitalba, A. glutinosa*, and *A. altissima* resulted positive, confirming their potential role as reservoir/source of infection for new outbreaks. New potential insect vectors were identified as *Phlogotettix cyclops* (Mulsant & Rey) and *Psylla alni* (L.) in Austria and *Oncopsis alni* (Schrank) in Slovenia.

Moreover, a new distribution map of FD strains in grapevines and other hosts have been designed, including previously undescribed strains with 'mixed profiles' identified in Italy and Austria.

Validation of diagnostic procedures. Validated protocols and panel of tested samples are detailed in The EUPHRESCO GRAFDEPI GROUP (2015). Comparative tests showed that the majority of the qPCR protocols tested had a SE and SP higher than 90%, (Table 2). The results recommend the use of qPCR methods.

Design of surveillance system schemes. General rules to be used for designing of surveillance systems based on new and latest epidemiologic data were provided with regards to sampling plan (period, number of samples, matrices, etc.), diagnostic protocols, monitoring and vectors distribution. For FD sampling and inspection of nurseries two strategies based on different risk factors were evaluated and proposed: a) sampling designs for randomly selected samples/nurseries and b) sampling designs for risk based selected samples/nurseries. The qPCR based procedures were found reliable and suitable for a sensitive and specific detection of the FD phytoplasmas and their use is recommended compared to the so far available protocols for FD detection and identification (EPPO PM7/79). Finally, indicators which allow the early detection of the occurrence of *S. titanus* and FD should be considered in novel control strategies, in addition to a systematic risk analysis. These indicators can be derived from intensive monitoring that should encompass also a specific larvae monitoring and testing of latent infections in high risk areas.

Table 1. List of participating Institutions

Institution	Contact	Country
CRA-PAV, Plant Pathology Research Centre	G. Pasquini*	Italy
AGES, Austrian Agency for Health and Food Safety	H. Reisenzein	Austria
CRA-W, Walloon Agricultural Research Centre	S. Steyer	Belgium
PPRS, Plant Protection Research Station	N. Ustun	Turkey
INIAV National Institute of Agrarian and Veterinary Research	E. Sousa, E. Silva	Portugal
ACW, Agroscope Changins-Wädenswil	S. Schaerer	Switzerland
ILVO, Institute for Agricultural and Fisheries Research	K. De Jonghe	Belgium
DipSA, *Alma Mater Studiorum*, University of Bologna	A. Bertaccini, S. Paltrinieri	Italy
Dipartimento di Produzione Vegetale, University of Milan	P. Bianco, P. Casati	Italy
CRA-ABP, Agrobiology and Pedology Research Centre	B. Bagnoli	Italy
IPEP, Institute of Pesticides and Environmental Protection	B. Duduk, J. Mitrovic	Serbia
NIB National Institute of Biology	M. Dermastia	Slovenia
IRTA, Institut de Recerca i Tecnologia Agroalimentaries	A. Battle	Spain
ANSES	M. Loiseau	France
CRA-VIT, Centro di Ricerca per la Viticoltura	E. Angelini	Italy

*Scientific coordinator of the Project

Table 2. Performance of methods for the detection of the 16SrV phytoplasma group.

	Universal nested PCR + RFLP	Group specific nested PCR	Duplex group specific nested PCR	Real-time PCR (Angelini *et al.*, 2007)	Real-time PCR (Hren *et al.*, 2007)	Triplex real-time PCR (Pelletier *et al.*, 2009)	Triplex real-time PCR (oligonucleotides under patent)
Genome target	16S rDNA	16S rDNA	SecY gene	16S rDNA	SecY gene	map gene	unknown
Nb of laboratories	5	13	12	7	10	6	7
Mean SE	88.9%	91.4%	83.7%	86.7%	97.3%	97.7%	100%
Mean SP	93.2%	88.3%	92.4%	66.1%	94.1%	93.3%	100%
100% positive results	< 1/10	< 1/10	< 1/10	< 1/10	< 1/10	1/100 - 1/2700	1/10 - 1/300
Reproducibility	67.7%	73.8%	60.2%	75.6%	84.9%	93.3%	86.7%
Repeatability	77.6%	81.6%	92.5%	88.0%	91.0%	94.9%	88.3%

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OP 53 - Molecular epidemiology of '*Candidatus* Phytoplasma solani' by multilocus sequence analsis

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INTRODUCTION

Bois noir (BN) is a grapevine disease that is associated to '*Candidatus* Phytoplasma solani' ('*Ca.* P. solani'; 16SrXII-A subgroup) (Quaglino et al., 2013), and it is most common and widespread in Euro-Mediterranean regions (Maixner et al., 2011), becoming a real limiting factor for the productions. In cv. 'Chardonnay', which is particularly sensitive to BN infection, the drying up of grape bunches can result in production losses of about 50%, with lower sugar content in the grapes of symptomatic plants (Endeshaw et al., 2012). '*Ca*. P. solani' isolates are characterised by different degrees of genetic variability according to the genes involved (Quaglino et al., 2009, 2013; Foissac et al., 2013). The most variable genes are those that code for surface membrane proteins phytoplasma, which are directly exposed to host and vector interactions. In particular a wide molecular characterization of stolbur isolates coming from different Countries of Mediterreanean Bacin revealed a high genetic variability of genes coding for variable membrane protein-1 (*vmp*1), and antigenic membrane protein (*stamp*) (Fabre et al., 2011; Foissac et al., 2013), which can be considered as suitable markers for molecular epidemiology. In this study, we combined data coming from genotyping by multilocus sequence analysis with estimation of the dN/dS ratio, which is the ratio between the non-synonymous (dN) and the synonymous (dS) substitution rates in an alignment of amino-acid-coding sequences (Nielsen, 2005), in order to estimate the richness of stolbur molecular genotypes and the pressure of selection within a highly BN infected commercial vineyard.

MATERIALS AND METHODS

The DNAs, extracted by CTAB protocol, were amplified in nested-PCR with specific primer pair for *tuf*, *vmp*1, *stamp* and *secY.* Then the *tuf and vpm*1 amplicons were digested in PCR-RFLP (Langer and Maixner, 2004; Fialova et al., 2009) in order to distinguish the molecular types. On the basis of the RFLP characterisation of *tuf* and *vmp*1 genes, representative samples within the vineyard, amplified with specific primer pairs for the *vmp*1, *stamp* and *secY* genes were purified and sequenced. The phylogenetic relationships were reconstructed, using the Mega v. 5.1 software, for the *vmp*1, *stamp*, and *secY* nucleotide sequences of '*Ca*. P. solani' that originated from the study vineyard, with respect to nucleotide sequences from other Italian regions and from Euro-Mediterranean countries that were available in Genebank. Moreover, the ratio between the proportion of non-synonymous and synonymous substitutions (dN/dS ratio), was determined for the nucleotide sequences at the study vineyard level in order to determine the type of selection interfere on *vmp*1 gene. Positive selection happens when dN/dS ratio >1.0, on the other hand a ratio <1.0 suggests purifying selection process (Nei and Kumar, 2000).

RESULTS AND DISCUSSION

In the study vineyard, the molecular characterization on the basis of the *tuf* gene revealed that the grapevines were mainly infected by *'Ca*. P. solani' *tuf*-type b (92.3%), with occasional *tuf*-type a infections (7.7%). As previously reported by Murolo et al. (2010), in the Marche region, nettle is rarely found around vineyards, and the BN-infected grapevines showed mainly (~80%) *'Ca*. P. solani' *tuf* type b. The molecular characterization of *vmp*1, allowed to detect eight different *vmp*1 types (V3, V4, V9, V11, V12, V14, V15 and V18), most of them identified in *H. obsoletus* collected in Marche vineyard ecosystems (Landi et al., 2015). The wide genetic diversity of *'Ca*. P. solani' has been reported and generally related to complex interactions between the vector and the wide range of wild host plants (Kessler et al., 2011). The dominant *vmp*1 genotypes were V14 and V12, while sporadically we detected V3.

The phylogenetic analysis was carried out on nucleotide sequences, which were representative of the RFLP types of the study vineyard, and on those available in GenBank. In the resulting dendrogram, the sequences generally clustered according to the PCR-RFLP patterns. Strains with the same RFLP pattern showed high nucleotide similarity (>99%) of sequences (Murolo *et al*., 2010). The selective pressure in the *vmp*1, *stamp* and *secY* genes were estimated for the 'Ca. P. solani' strains according to the abundance of non-synonymous mutations. For the *secY* gene, the overall dN/dS ratio was 1.02 (P =0.841), which suggested low neutral selection across this gene. The overall ratio between the non-synonymous to the synonymous mutations (dN/dS) was >1.0 for *vmp*1 (2.28; P = 0.001) and *stamp* (3.99; P = 0.019). These high values

of dN/dS (i.e., >1) indicated detection of a high number of non-silent (dN) mutations. The higher genetic variability in the *vmp*1 and *stamp* genes with respect to the *secY* gene arose from the estimation of the rate of non-silent mutation (dN). According to this parameter, which is an indication of selective pressure, Fabre et al. (2011) defined the *secY* gene as a housekeeping gene, while the *vmp*1 and *stamp* genes were under positive selection, because they are involved in specific interactions as demonstrated for other phytoplsma (Kakizawa et al., 2006a). The high genetic variability as well as the dN/dS ratio >1 of '*Ca.* P. solani' in *vmp*1 and *stamp* genes, within a restricted location (i.e. commercial vineyard) provide useful information to trace an inoculum source and the movement of pathogen strains over local and long distances (Murolo and Romanazzi, 2015).

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OP 54 - Field study of the interaction of nettle, the "bois noir" vector *Hyalesthes obsoletus,* and '*Candidatus* Phytoplasma solani'

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INTRODUCTION

"Bois noir" of grapevine is associated with infection by "stolbur" phytoplasma (StolP), '*Candidatus* Phytoplasma solani' and principally transmitted from alternative plant hosts to grapevine by species of Cixiidae, with *Hyalesthes obsoletus* as the most common vector. "Bois noir" is characterized by temporal fluctuation of disease incidence which is related to the variation of infection pressure that results in short epidemic periods followed by several years with low infection pressure and decreasing disease incidence (Maixner, 2006). Which factors drive this variation are still unknown. Herbaceous wild plants serving as hosts for both the vector and the pathogen are key factors for infection pressure. Stinging nettle (*Urtica dioica*) growing along vineyard borders or on fallow patches is an important source of StolP-inoculum (Bressan *et al*., 2007; Mori *et al*., 2012). The immature vectors acquire the pathogen in spring during feeding on the roots of infected nettle (Kaul *et al*., 2009). In this study the interaction of nettle host plants with *H. obsoletus* and StolP was monitored in a small scale comparing individual nettle stands over three years with the objective to understand the variation in infection pressure to grapevine.

MATERIALS AND METHODS

The study was carried out on a fallow plot of approximately 1,600 m^2 at a viticultural site in the Mosel valley, Germany, from 2011 to 2013. Some results of the first year have been reported before (Maixner and Johannesen, 2012). Nettle stands were mapped and measured every year. They were divided in two cohorts: bushes already present in spring of 2011 ("old") and those that newly developed during 2011 and were first sampled in 2012 ("new"). The plot was mowed once a year in spring except for the nettle bushes. Root samples (3 to 14 per bush depending on bush size) were taken in 2011 and 2013 for phytoplasma detection in nettle. Each nettle bush was sampled separately for *H. obsoletus* throughout the flight periods of the vector by sweep-net. Five to 60 sweeps per bush were carried out depending on bush size. To compare collections between years the data were standardized by dividing the total number of individuals per bush by the number of samplings and sweeps. Fifty insects per bush were analyzed each year by PCR for StolP presence.

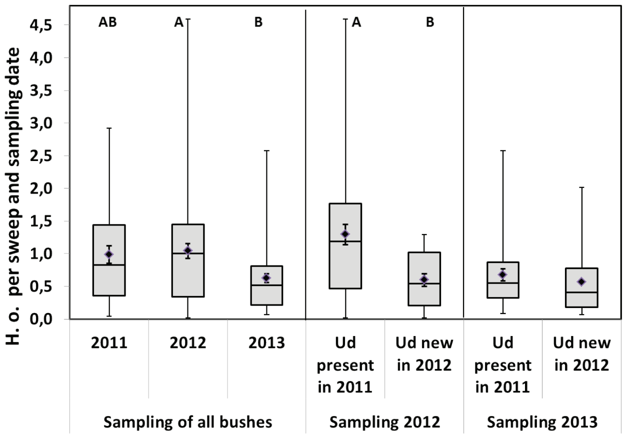
RESULTS AND DISCUSSION

The number of nettle stands increased over the years (31, 52, 60, respectively), probably because nettle was favored by mowing of the accompanying vegetation. The average bush size declined, however, from 1.5 m^2 to 1 m^2. The ratio of PCR-positive root samples was 83% in 2011 but it declined significantly to 34% in 2013 (X^2=25,8; d.f.=1; p<0.001) although the proportion of infected bushes (at least one infected root sample per bush) did not change significantly. The infection rate in "old" bushes was still higher than in "new" bushes in 2013 (45% vs. 17%; X^2=10.0; d.f.=1; p<0.002).

The sampling of *H. obsoletus* over three years yielded 26,300 individuals. The flight period lasted between 83 (2011) and 70 (2013) days. The vector's overall density did not differ between 2011 and 2012 [0.99 vs. 1.02 individuals/(sweep x sampling) (iss)], but declined significantly in the last year (0.62 iss) (Fig. 1A). Whether this reduction was linked to the reduced average bush size in 2013 is not clear since a significant correlation between bush size and *H. obsoletus* density was only observed in 2012. Weather conditions during the development of the 2013 population (September 2012 to May 2013) might have had an adverse effect on the xerothermic vector species, since the average soil temperature was 2°K lower than in previous years while rainfall was raised by one third. The colonization of "new" bushes was not yet completed in 2012, because the vector's density was significantly lower compared to "old" bushes (0.6 iss vs. 1.3 iss; t=3.47, d.f.= 51; p<0.001), while the density of *H. obsoletus* from both cohorts was almost the same in 2013 (Fig. 1A).

The overall proportion of infected vectors was 12.8% in 2011. It was within the range of rates that are commonly observed in nettle populations of *H. obsoletus,* but the infection rate varied between years (ANOVA, F=13.87; d.f.=2; p<0.001). It dropped significantly to 3.8% in 2012 and 6.3% in 2013 (Fig. 1B). This corresponds to the reduced proportion

of nettle root samples in 2013 compared to 2011. However, in 2012 the infestation of vectors collected from "old" bushes was significantly lower than the one on "new" stands (2.4 iss vs. 7.4 iss; t=3.66, d.f.=52; p<0.001) although the tests of root samples revealed higher infection rates in "old" bushes. This apparent contradiction could potentially be explained by a preference of infected *H. obsoletus* for non-infected nettle plants. Other sources of infection beside nettle can be excluded since nettle is colonized by a monophagous host race of *H. obsoletus* in Germany (Imo *et al*., 2013).



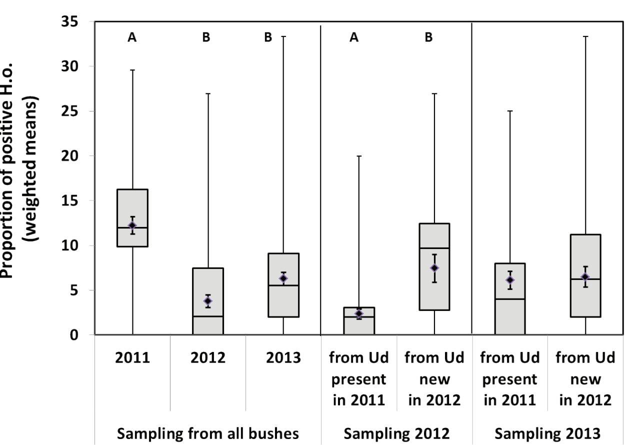


Figure 1: A Density of *H. obsoletus* on nettle bushes. **B**: Infection rates of *H. obsoletus* from nettle bushes. Boxplots: Median with upper and lower quartile, whiskers show minimum and maximum values. Diamonds with error bars represent the mean with standard error. Significant differences are indicated by different letters.

The interaction of nettle host plants, vectors and phytoplasma is not static, since significant changes in infection rates of both the nettle host plant and the vector populations were observed during a three year period. While the decreased vector density in 2013 was probably the result of unfavorable weather conditions, the drop of infection rates in 2012 and 2013 could not be linked to environmental parameters or biotic factors. To identify the determinants that drive the fluctuations of infection pressure by "bois noir" to grapevine, extended time periods covering both endemic and epidemic stretches of "bois noir" disease should be analyzed.

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OP 55 - Incidence and distribution of aster yellows disease of grapevine in the Olifants River wine producing area of South Africa

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INTRODUCTION

Aster yellows phytoplasma (AY, 16SrI-B group), was recorded for the first time in grapevine (*Vitis vinifera* L. (Vitaceae)) in South Africa in 2006 (Engelbrecht *et al*., 2010). Initially the disease occurred on grapevines in two regions, Olifants River and Wabooms River, but recently it was also found near Robertson, Trawal and Montagu in the Western Cape Province. An indigenous leafhopper, *Mgenia fuscovaria* (Stal), was identified to transmit AY to grapevine (Krüger *et al.*, 2011). Worldwide phytoplasma diseases of grapevine cause serious damage ranging from lower yields (20-30% but sometimes as high as 80%) to the death of vines (Magarey, 1986).

The aim of this study was to conduct surveys in disease-affected vineyards in the Olifants River area to determine the incidence and distribution of the disease in a variety of cultivars. This epidemiological study gives an indication of the tempo of spreading and the potential impact of the disease on the South African wine industry. It will also contribute valuable information towards the development of a management strategy for grapevine yellows disease in South African vineyards.

MATERIALS AND METHODS

Surveys to determine AY disease incidence were conducted annually from 2009 to 2013. Intensive disease mapping were performed, recording both the disease status and spatial location of all vines in a vineyard. Disease incidence assessments were conducted during late summer (late January or early February) just before harvest, when symptoms were most apparent. Each vine was characterised as healthy, AY affected or missing/dead. Vines were considered AY-affected if any one of the following visual symptoms of the disease were present: (1) aborted bunches, (2) downward rolling and yellowing/reddening of leaves, (3) green, immature canes and/or (4) die back of shoot tips and shoots. The yearly incidence (%) was determined for each vineyard (= number of vines showing disease symptoms in the current year), as well as the cumulative incidence (%) (= sum of all new records of grapevines showing disease symptoms in the current year and all records of diseased grapevines in previous years). Statistical analyses were performed on the disease incidence data of 7 vineyards from 3 cultivars (Chardonnay, Chenin blanc and Pinotage), which were all surveyed for 4 years. Student's t-least significant difference was calculated at the 5% level to compare treatment means. A probability level of 5% was considered significant for these tests.

In order to confirm visual symptom evaluation of vines, five symptomatic and five asymptomatic vines were annually sampled per vineyard and subjected to diagnostic PCR analysis. Total nucleic acid was extracted from leaf veins according to Angelini *et al*. (2001). The presence of AY phytoplasma was determined by using PCR-RFLP, as described by Lee *et al*. (1998), using restriction enzymes *Alu*I, *Hha*I, *Hpa*I and *Rsa*I. Nested PCR was performed using two sets of universal primers (P1+P7, followed by R16F2n/R16R2). Additional restriction enzymes, namely *Kpn*I, *Taq*I and *Tru1*I were included from the second season onwards.

The PATCHY spatial analysis package (Maixner, 1993) was used to test for randomness or clustering by ordinary runs analysis.

After the identification of the insect vector, *Mgenia fuscovaria* (Stal) in 2010, producers treated vines with the systemic neonicotinoid insecticide, imidacloprid. All vineyards in the survey were treated with imidacloprid in the spring of 2010 and after harvest in March 2012.

RESULTS AND DISCUSSION

Varied disease incidences were recorded for the different cultivars studied, namely Chardonnay, Chenin blanc and Pinotage, and incidences also varied between the different sites. Pinotage showed a mean yearly disease incidence of 5.8%, which was lower than Chenin blanc (16.64%) or Chardonnay (29.95%). However, statistically there was no significant

difference ($p \leq 0.05$) between the mean yearly disease incidences of the three cultivars. Disease incidences of five vineyards showed an increasing pattern and in two of the vineyards yearly disease incidence fluctuated. These fluctuating patterns could be contributed to pollarding of vines or objectivity of the evaluating team. Cumulative incidences indicated that new records of AY infected grapevines occurred in every vineyard in every year, except for one vineyard (Pinotage), which had no new infections in 2012. There was no significant difference between the quantity of new AY infections occurring in the three different cultivars. Although the mean cumulative disease incidence of Pinotage (10.87%) was lower than that of Chenin blanc (32.31%) and Chardonnay (37.77%) the mean cumulative disease incidence of the three cultivars did not differ significantly. Disease incidence of AY could be potentially higher than what was found in this study if no control of vectoring insects was performed and natural spread of the disease was allowed.

Symptomless AY phytoplasma infections were found to occur in South African grapevines and AY phytoplasma could not be detected in all symptomatic vines which confirm the uneven distribution of the phytoplasma in vines as previously described by Constable *et al*., (2003).

Spatial distribution patterns were non-random with clustering occurring along and across vine rows in most of the vineyards surveyed. Aggregation of infected vines mostly occurs on the side of vineyards adjacent to infected vineyards.

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OP 56 - Successful elimination of Grapevine rupestris stem pitting-associated virus and its gradual re-infection in the vineyard but not in the greenhouse

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INTRODUCTION

Grapevine rupestris stem pitting-associated virus (GRSPaV, genus *Foveavirus*, family *Betaflexiviridae*) is the most commonly occurring virus in the grapevine worldwide. Over 90% of the grapevines in Australia are infected with this virus. It is one of the most diverse species in the family with nine molecular variants (Terlizzi et al., 2011; Martelli, 2014). It exists as quasispecies in a single plant, often symptomless, but a few strains are associated with stem pitting and possibly vein necrosis (Bouyahia et al, 2005). The mode of transmission of this virus in vineyards is not known. Seed and pollen transmission have been reported but could not be confirmed (Rowhani, 2000; Martelli, 2014). Attempts to eliminate GRSPaV by routine tissue culture and thermotherapy methods proved difficult (Gribaudo et al., 2006). By eliminating GRSPaV from the grapevine we will be able assess its effect on berry production and wine quality by comparing virus-free with infected individuals. As part of our virus elimination program *Vitis* varieties infected with GRSPaV were subjected to thermotherapy and after successful elimination of the virus, these were either planted in the vineyard or left in the greenhouse for virus-indexing. We present the results of indexing for GRSPaV over the period 2009-2015.

MATERIALS AND METHODS

For virus elimination, shoots from the following varieties known to be infected with GRSPaV were sampled in 2008: *Vitis vinifera* "Chardonnay", "Nebbiolo", "Arnies", "Sangiovese" and "Ramsey" rootstock (*Vitis champini*). Shoot tips of 1-2 mm size were sterilized and placed on a growth medium (Murashige and Skoog, 1962). For thermotherapy, the tips were cultured in a growth cabinet at temperatures which were gradually increased from 25/20 °C (day/night) to as high as 42/36 °C over a period of 15 weeks. Three random explants were collected every fortnight for virus detection. At least six virus negative explants from each variety were transferred to rooting media and then to a greenhouse. In the spring of 2009 half of the plants were transferred to our vineyard and the rest were left in the greenhouse. Total nucleic acids were extracted either from *in vitro* grown tissue or from the phloem shavings of dormant canes essentially according to the protocol described by MacKenzie et al. (1997). Single tube RT-PCR was used for virus detection using two pairs of primers. The routine primer pair was RSP48 (5'AGCTGGGATTATAAGGGA GGT) and RSP49 (5'CCAGCCGTTCCACCACTAAT) which produced an amplicon of 329 bp from the CP gene (Zhang et al., 1998). The second pair was generic RSP35 (5-AGRYTTAGRGTRGCTA ARGC) and RSP36 (5'CACATR TCATCVCC YGCAAA3) targeting the RdRP gene (Terlizzi et al, 2011). This primer pair produces a PCR product of 478 bp which can detect a total of seven geno-groups of GRSPV (B. Meng personal communication). Amplicons primed with RSP 48/RSP49 were *cloned into* the *pGEM-T Easy vector* and sequenced using universal M13 forward or reverse primers (AGRF, Adelaide, Australia).

RESULTS AND DISCUSSION

An amplicon of 329 nt from the CP gene was sequenced to confirm that a segment of GRSPaV CP had been targeted (Habili et al., 2006). This GRSPaV amplicon showed a similarity of 98% at the nucleotide level and 100% at protein level with a type isolate of the virus (Acc. No. KJ634652). The survey presented here is based on the detection of this segment of the CP. This was confirmed by using the generic primer pair of Rsp35/RSP36 on the RdRp. Tissue cultured plants free of the specific GRSPaV amplicon were either maintained in the greenhouse or transferred to our vineyard which had an infection rate of 95% (unpublished). The indexing results showed that all the plants growing in the greenhouse remained negative for the virus up to 2015. On the other hand, those grown in the vineyard showed an increasing number of virus positive signals each year. In 2015, there was only one plant which was still negative (Table 1, vine 11). Root-grafting was not involved in the spread as the emergence of positives among plants was random and rapid, especially towards the final years (Table 1). Likewise, it appears that pollen did not have any role in the spread as four of the positive plants had not reached the flowering age (Table 1).

Table1. Re-infection of GRSPaV following transferring virus eliminated vines to the vineyard[1]

Plant No. in the Row	Variety	2009	2012	2013	2014	2015
1(south)	Nebbiolo	Negative	Negative	Negative	Positive	Positive
2	Nebbiolo	Negative	Negative	Negative	Negative	Positive
3	Arnies	Negative	Negative	Positive	Positive	Positive
4[2]	Ramsey-A[3]	Negative	Negative	Negative	Positive	Positive
5	Ramsey-A	Negative	Positive	Positive	Positive	Positive
6	Ramsey-A	Negative	Negative	Positive	Positive	Positive
7	Ramsey-B	Negative	Negative	Positive	Positive	Positive
8	Ramsey-B	Negative	Negative	Positive	Positive	Positive
9	Ramsey-B	Negative	Negative	Positive	Positive	Positive
10	Chardonnay	Negative	Negative	Negative	Positive	Positive
11	Chardonnay	Negative	Negative	Negative	Negative	Negative
12	Sangiovese	Negative	Negative	Positive	Positive	Positive
13	Sangiovese	Negative	Negative	Negative	Negative	Positive
14 (north)	Sangiovese	Negative	Negative	Negative	Negative	Positive
Total +ves:		0	1	7	10	13

[1]Duplicate plants left in the greenhouse remained virus-free. [2]Numbers in bold refer to the plants that have not reach the flowering stage (May 2015) but tested positive. [3]Two clones of the "Ramsey" rootstock (*Vitis champini*) were used.

In California, efforts to eliminate GRSPaV from vines have been hampered by an unexplained spread of the virus in vineyards (http://iv.ucdavis.edu/Viticultural_Information/?uid=115&ds=351).

Here, we conclude that spread of GRSPaV has occurred in our vineyard, and that containment in a glasshouse protects against reinfection. This result suggests that the high incidence of infection by GRSPaV in Australian grapevines may be due to natural spread of this virus. So far, no vectors have been identified for any foveavirus. Trials can be set up using this virus as a model system to test whether certain exclusion treatments might identify air-borne, soil-borne or pollen-borne modes of transmission. If a biological vector is identified for GRSPaV, virus control in the long term could be directed at the vector.

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OP 57 - Interpreting a north coast multi-virus survey in order to assess benefits of disease management

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INTRODUCTION

Grape production in the US accounted for $5.76 billion in 2014, predominantly in California (NASS, 2014). Virus diseases are a long-standing issue in grape production worldwide. Grapevines are asexually reproduced via cuttings, and viruses are easily spread via contaminated material (Maree et al. 2013), in addition to some insect transmitted viruses, like *Grapevine leafroll-associated virus-3* (GLRaV-3) (Golino et al. 2008). Grapevine leafroll disease (GLRD) alone can result in economic losses ranging from $29,902 to $226,405 depending on the wine growing region (Ricketts et al. 2015). In California, many grape producers utilize certified, virus tested nursery stock (planting material) when establishing vineyard blocks. The Grape Registration and Certification program can benefit the industry as much as $57 million per year in terms of GLRaV-3 (Fuller, Alston, and Golino 2015). Advances in virus detection, such as next generation sequencing (NGS), have led to the discovery of new grape viruses, namely *Grapevine redblotch-associated virus* (GRBaV) (Al Rwahnih et al. 2013; Al Rwahnih, Rowhani, and Golino 2015). While there are more than 70 known virus and virus like diseases of grapevines, GRBaV, a cryptic (hidden) virus, and GLRaV-3, a vector transmitted virus (Al Rwahnih, Rowhani, and Golino 2015; Martelli 2014; Daane et al. 2012; Tsai et al. 2008; Petersen and Charles 1997) question the effectiveness of the Grape Registration and Certification program. In order to assess this concern and determine virus reservoirs in vineyards; an area wide survey is needed that takes into account planting booms (large influxes of nursery material moving into production) in relation to infected planting material entering and exiting the supply chain.

MATERIALS AND METHODS

An ecological survey was performed in commercial vineyards in the north coast region of California in order to evaluate the incidence of a range of grapevine viruses throughout “planting booms”. Sample collection occurred from August to October of 2014. The structure of the survey incorporated 27-29 vineyard blocks from each of four different planting eras: 1880-1980, 1981-1995, 1995-2010, and 2011-2014. Please see table 1 for descriptions. A blind, simple random sample was collected in a “W” formation across the block to obtain 5-15 (according to acreage) individual vine subsamples, symptomatic and non-symptomatic (Madden et al. 2007). Each vine sample consisted of 4 composite petiole or cane samples collected near the base of the shoot. Each subsample was assayed for the presence of *Grapevine leafroll-associated virus-1*,*-2*,*-3*, *Grapevine red blotch-associated virus*, *Grapevine vitivirus A*,*B*, *Grapevine fleck virus*, *Grapevine fanleaf virus*, and *Rupestris stem pitting-associated virus* (GLRaV-1, -2, -3, GRBaV, GVA, GVB, GFkV, GFLV, RSPaV respectively) using species-specific qPCR primers. A cycle threshold of 30 was used to determine positive signals (Klaassen et al. 2011).

Table 1. Treatments are shown as age ranges. Each treatment was surveyed at the block level (replications), subsample number collected based on the acreage of the block. Data were analyzed as proportions of positive signals per block.

1	1880 1980	Heritage material, predominantly not certified, many blocks replanted on AXR#1 rootstock (a hybrid rootstock of *V. vinifera* x *V. rupestris*) in response to grape phylloxera (*Daktulosphaira vitifoliae*, an aphid-like insect that is a root pest) epidemic (Wolpert et al. 1994)
2	1981 1995	Many blocks were removed and replanted due to the failure of AXR#1 to a potential divergent strain of phylloxera (phylloxera Type B) (Wolpert et al. 1994).
3	1996 2010	AXR#1 failure-related replacement vines were planted on rootstocks of American species parentage and coincidently susceptible/resistant to viruses contained in the scion material (symptoms previously masked by rootstocks such as St. George and AXR#1). Virus issues became apparent to industry and many vineyards were replanted with certified nursery stock (scion and rootstock).
4	2011 2014	The recession (2008-2009) left many blocks in fallow, or in lieu of replanting. Many blocks were planted/replanted with certified material and are under current GLRaV-3 management (rogueing infected vines, vector monitoring and control).

RESULTS AND DISCUSSION

Overall, virus incidence (for each virus assay) at the block level ranged from 0 to 100%, although only a subset of the nine viruses assayed were present in each block. The mean incidence values and standard deviation bars are shown graphically in figure 1.

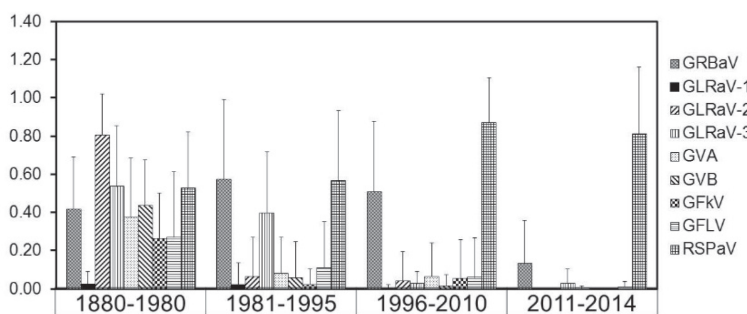

Figure 1. Mean incidence levels for grapevine viruses detected in a stratified random sample of north coast grape blocks in the 2014 growing season. Planting year of block is expressed on the *x*-axis, virus incidences are expressed as proportions on the *y*-axis. Analysis of the frequency distribution of each virus proportion in each treatment is shown in figure 2.

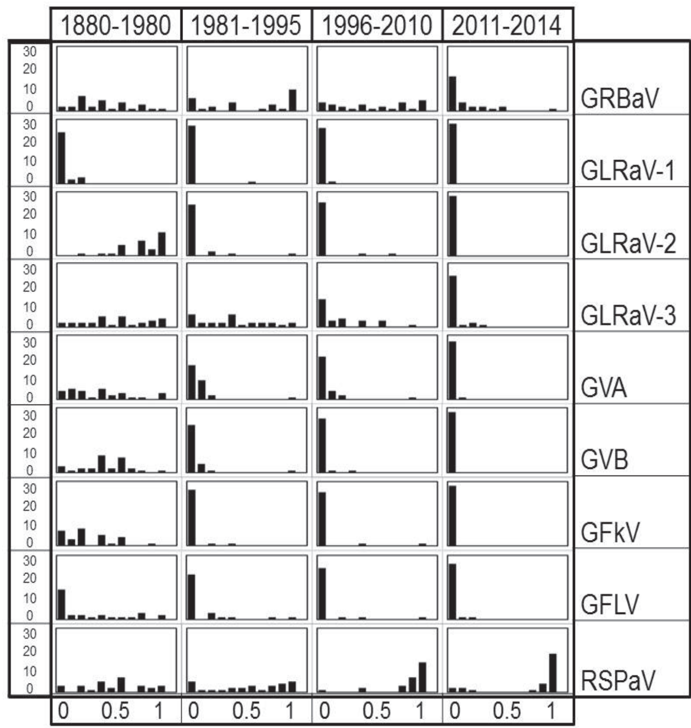

Figure 2. Frequency of virus incidence is expressed on the *x*-axis, virus incidence per block is expressed on the *y*-axis, virus assay is described to the left of the figure, treatment (planting boom) is defined above the figure.

Frequency distributions of virus incidences in material appear evenly distributed in heritage material, 1880-1980. Distributions appear to shift towards 0% as material is renewed, except for RSPaV, which is not currently in the certification program because detriment to the vine has not been demonstrated and transmission is not well understood (Weber, Golino, and Rowhani 2002). Additionally GRBaV is not included in the certification program, but growers are currently managing GRBaV in north coast vineyards via rogueing and screening of plant material.

The perceived temporal pattern of virus incidence is indicative of the impact of the grape nursery stock certification program and regionally based virus management. Specific viruses show shifts in their incidence distribution toward lower mean values as the certification program grew in importance.

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OP 58 - Towards the definition of the *absolute* sanitary status of certified grapevine clones and rootstocks

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INTRODUCTION

The production of certified grapevine propagation material relies on woody indexing (WI) and laboratory tests. The former is a tedious, costly and time-requiring procedure. In a recent paper (Constable *et al.*, 2013) a minimum period of three years of observations in the field is recommended, because of WI dependence on climatic conditions and frequent graft failures. Furthermore, a standardized assay protocol is lacking, thus WI is often performed in different ways and conditions. The consensus is that all certification systems, regardless of the country of implementation, require WI for diseases like leafroll, infectious degenerations, rugose-wood and fleck. In parallel, laboratory tests in which different reagents and protocols are used, are performed for detecting the viruses known as the agents of these diseases. Due to their specific design, both types of tests fail in identifying new, unknown agents. Because of these limitations, the adoption of a "common language" in defining the sanitary status of plant propagation material would be desirable. This is now an accessible objective thanks to the availability of high throughput techniques for sequencing (HTS), which are not reliant on the extant knowledge of infectious agents (viruses and viroids) and on the extreme specificity of laboratory assays (serological and molecular). Data originating from HTS give an unbiased snapshot of the virome of any given vine and can universally be shared for commercial, quarantine and scientific purposes.

We have applied these techniques to investigate the virome of a group of commercial clones of grapevine cultivars and rootstocks whose sanitary status had previously been defined by WI and laboratory assays according to the Italian regulation for the production of certified grapevine plant propagation material.

MATERIALS AND METHODS

Grapevine sources: Twenty clones of grapevine cultivars and rootstocks were selected for this study. These clones, which had undergone sanitation procedures, were known to be free from viruses and diseases regulated by the Italian scheme for the production of certified grapevine propagation material, as assessed by WI and laboratory assays. The clones, all of "basic" category, were maintained in the premultiplication block of CRSFA, Centro di Ricerca, Sperimentazione e Formazione in Agricoltura "Basile Caramia", Locorotondo (Bari), Italy.

Libraries preparation and analysis: Purified small (sRNAs) from leaf or phloem tissues were used to synthesize cDNA libraries according to an optimized version of Illumina protocol described by Giampetruzzi *et al.* (2012). A 50 base-single read run was done on a HiScan SQ™ apparatus. Short sequences were processed with a customized bioinformatic pipeline as in Giampetruzzi *et al.* (2012).

Validation of HTS data by RT-PCR: Total RNA extraction and cDNA synthesis were done according to the validated protocol described by Faggioli *et al.* (2012). PCR detection was performed with primers designed by Gambino and Gribaudo (2006), used in a single, instead of multiplex reaction, for *Grapevine virus A* (GVA), *Grapevine virus B* (GVB), *Grapevine leafroll-associated virus 1, 2, 3* (GLRaV-1, -2, -3), *Grapevine fanleaf virus* (GFLV), *Grapevine fleck virus* (GFkV) and *Arabis mosaic virus* (ArMV), and by Zhang *et al.* (1998) for *Grapevine rupestris stem pitting-associated virus* (GRSPaV).

RESULTS AND DISCUSSION

Overall, the analysis of HTS data confirmed the healthy sanitary status of the 20 grapevine clones, which were free from GLRaV-1, -2 and -3, GFLV, ArMV, GVA, GVB and GFkV (Table 1). These findings were validated by RT-PCR analysis except for the non-regulated GRSPaV, which was detected by HTS and in two cases (V.17, V.6) had escaped RT-PCR. The high sensitivity of HTS, already reported by Hagen *et al*. (2012) or problems stemming from the extreme specificity of the primers could explain the better performance of this technique although more data are necessary to define substrates (i.e. double stranded RNAs, small RNAs or total RNAs), protocols, bioinformatics pipeline and minimal depth of data to be considered significant. In our experience a minimum of 3.302.822 raw reads, corresponding to sRNAs from leaf or phloem tissues, were sufficient to describe the virome of the analyzed grape with respect to certification-regulated viruses. The unbiased peculiarity and the HTS potential to discover new viruses is also proved by the finding of a new badnavirus

(Chiumenti *et al.*, 2015) apparently similar to Grapevine Roditis leaf discoloration-associated virus (GRLDaV).

A recent study of Al Rwahnih *et al.* (2015) highlighted the potential benefits of using HTS in grapevine certification schemes, leading the authors to envisage the possibility of substituting bioassays with HTS techniques. In addition to a substantial gain of time and costs, the adoption of a HTS approach would be helpful for the harmonization of certifications schemes among countries and for the commercial exchange of propagation materials. Providing HTS data specific for each grapevine clone could represent a sort of “pedigree” with a significant added value and guarantee for the grapevine industry. Our experience is a first step toward the establishment of an “absolute” sanitary status of grapevine plant propagation material.

ACKNOWLEDGEMENTS

This work was supported by Re.Ge.Vi.P. project (“Recupero del germoplasma viticolo pugliese”), funded by Regione Puglia, Italy, in the frame of the PSR 2007-2013, Measure 214, action 4a, “Progetti integrati per la Biodiversità”.

ID.	Cultivar/ Rootstocks	Clone code	Redundant reads (adpt. trimmed)	Contigs	Regulated viruses[a]		GRSPaV	
					NGS	PCR	NGS	PCR
V.1	Uva di Troia	UBA 49M	16.400.133	2.042	-	-	+	+
V.2	Malvasia Nera	UBA 69E	3.472.517	1.152	-	-	+	+
V.5	Bombino Nero	CRSA Reg. Puglia D205	22.872.057	1.152	-	-	+	+
V.7	Aglianico	CRSA Reg. Puglia D382	23.426.017	4.652	-	-	+	+
V.13	Baresana Rossa	CRSA 203	8.276.729	2.274	-	-	-	-
V.14	Italia	CRSA 121	23.332.063	5.477	-	-	-	-
V.15	Vittoria	CRSA 41	22.691.173	3.716	-	-	+	+
V.17	Regina dei Vigneti	CRSA 76	21.841.038	6.124	-	-	+	-
V.18	Lattuario Nero	CRSA 277	3.302.822	302	-	-	-	-
V.4	Verdeca	UBA 6A	12.128.430	3.173	-	-	+	+
V.6	Susumaniello	CRSA Reg. Puglia D382	8.607.208	4.524	-	-	+	-
V.8	Bombino Bianco	CRSA Reg. Puglia D382	14.014.780	2.351	-	-	+	+
V.10	Negramaro	CRSA Reg. Puglia D382	19.435.282	5.281	-	-	+	+
V.11	Regina Bianca	CRSA 11	6.066.427	1.640	-	-	+	+
V.12	Michele Palieri	CRSA 229	7.228.556	2.534	-	-	+	+
V.20	Kober 5BB	UBA 01	15.016.373	3.415	-	-	+	+
V.22	1103 Paulsen	UBA 08	21.236.510	9.510	-	-	+	+
V.23	140 Ruggeri	UBA 05	19.737.418	11.377	-	-	+	+
V.24	420 A Mill.de Gr.	UBA 08	14.990.088	9.133	-	-	+	+
V.25	110 Richter	UBA 05	13.619.296	8.089	-	-	-	-

Table 1. Results of NGS and RT-PCR analyses on certified grapevine cultivars and rootstocks. [a] GVA, GVB, GLRaV-1,-2,-3, GFLV, GFkV and ArMV, according to Italian (DM 07/07/2006 and DM 24/06/2008) regulations. Light and dark grey indicate extractions from leaf or phloem tissues, respectively.

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POSTER PRESENTATIONS

PP 01 - Molecular characterization of Grapevine fan leaf virus from non Vitis hosts

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INTRODUCTION

Grapevine fanleaf virus (GFLV) (a member of the genus *Nepovirus*, family *Secoviridae*) is responsible for the for an economic disease of grapevines throughout the world (Andret-Link *et al.,*2004). It is naturally transmitted by the soil nematode *Xiphinema index*, with the coat protein determining transmission specificity (Schellenberger *et al.,*2011). Symptoms of the disease include fanleaf, mosaic, shortened internodes and in chromogenic strains severe leaf chlorosis. GFLV naturally infects grapevine (Andret-Link *et al.,*2004) as well as Bermuda grass and knotweed in Iran (Izadpanah *et al.,* 2003a, 2003b).

Despite ample information on molecular variability of GFLV isolates from grapevine, little is known about molecular aspects of this virus from other hosts. In this paper, we report new weed hosts of GFLV and . molecular variability of GFLV isolates from non-vitis hosts.

MATERIALS AND METHODS

Samples of grapevine and herbaceous plants were randomly collected from vineyards of Iran during growing season of 2012-2014.Total RNA was extracted from the samples using CTAB-PVPP method (*Gibbs* and *Mackenzie 1997*). cDNA was synthesized using reverse transcriptase (*RevertAid*™) and oligo-dT primer. PCR reactions were performed in a final volume of 25µl with *Taq* DNA polymerase (Amplicon Red PCR master mix, Denmark), using a primer pair designed in this work (5'GGATTAGCTGGTAGAGGAG3'/5'CACAAACAACACACTGTCGCC3'), based on sequence of the Iranian isolates of GFLV, targeting the capsid protein (CP) gene. Amplicons were ligated into InsT/A Clone PCR Product Cloning Kit (Fermentas,Thermo Scientific, Inc.) and transformed to *E. coli* XLBlue competent cells. Recombinant plasmids were purified from bacterial cells using Prime Prep Plasmid DNA Isolation Kit (Genetbio-Korea). Recombinant clones were sequenced in both directions. The sequences were aligned using Muscle and phylogenetic trees were constructed using maximum likelihood algorithm performed with MEGA version 5.1.

RESULTS

A fragment of 1515bp was amplified from the herbaceous and grapevine samples by RT-PCR. Bermuda grass, Knotweed, Johnson grass, Raspberry, *Melilotus* sp., *Plantago lanceolata* were found to be naturally infected with GFLV .

Pairwise alignment of the sequences revealed 79-99% identity of herbaceous isolates with grapevine isolates of GFLV at nucleotide and amino acid levels . *Rubus* isolates of the virus were the most divergent.

In the maximum likelihood tree the Iranian isolates of GFLV formed a distinct cluster. They consisted of two sister clades of North East and North West isolates reflecting their geographical separation.

Herbaceous isolates from Bermuda grass, knotweed, Johnson grass, *Melilotus* and *Plantago* and *Rubus* plants from North West and *Rubus* isolates of Sothern Iran were closely related to grapevine isolates from North West of Iran. Surprisingly, Bermuda grass isolates of Sothern Iran showed similar properties to grapevine isolates of other countries (Fig 1).

DISCUSSION

Bermuda grass, knotweed, and raspberry were reported previously as non-Vitis hosts of GFLV in Iran (Izadpanah *et al.,* 2003a, 2003b), Here we report Johnson grass, *Melilotus* sp. and *Plantago lanceolata* as new hosts of GFLV in this country.

Previous analyses based on the MP and CP genes of GFLV have demonstrated that Iran GFLV isolates have distinct

phylogenetic position (Sokhandan-Bashir *et al.,* 2007, 2009). Weed isolates of GFLV show similar molecular properties to grapevine isolates. Also, geographical isolation has significant effect on their phylogenetic relationships. The level of genomic variation suggests that GFLV genomes may consist of a genetically diverse collection of variants, in the manner of a quasispecies (Roossinck 1997). Surprisingly we found two distinct populations of GFLV among Iranian isolates. A divergent isolate showed different evolutionary pathway and formed a separate clade in the phylogentic tree, whereas the others had similar properties to GFLV-F13. This gives further support to the previous hypothesis that GFLV has originated in this region from where it has spread to other parts of the world (Vuittenz1970).

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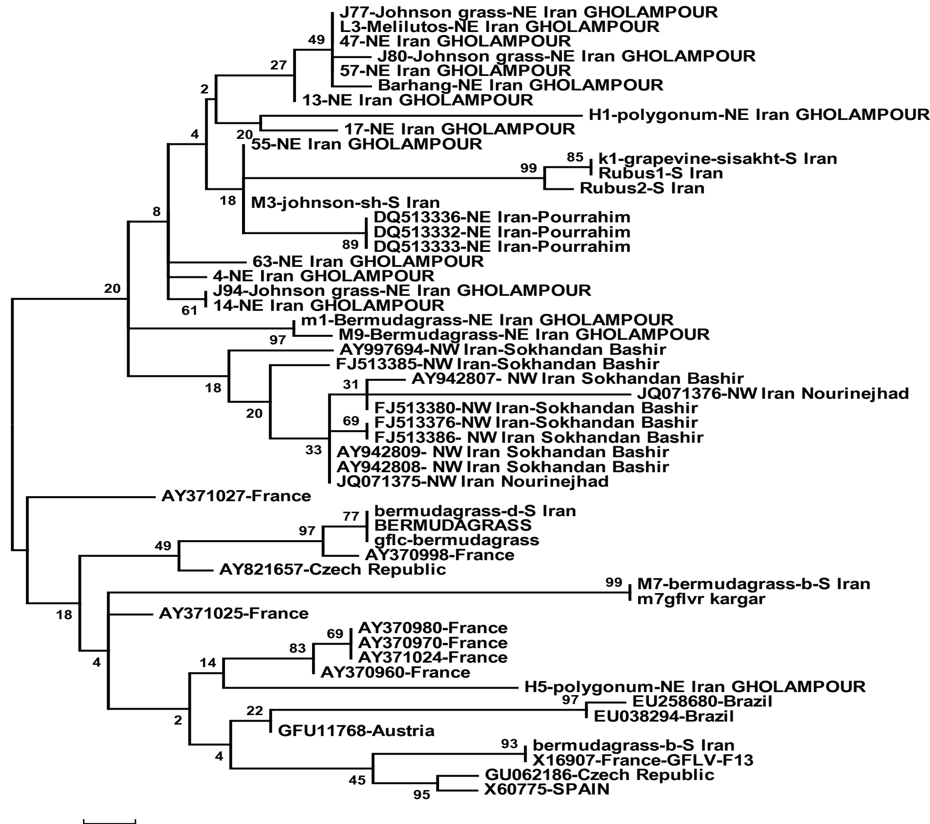

Figure 1: Maximum likelihood tree represent phylogenetic relation of weeds isolates of GFLV with other isolates of GFLV

PP 02 - Grapevine virus diseases testing in the seedlings introduced to Ukraine

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INTRODUCTION

Testing of imported to Ukraine clonal and regular grapevine seedlings in laboratory conditions is very important, because it allows to detect the virus diseases that may be present in the seedlings and thus to prevent their distribution. Today most dangerous grapevine viruses may be detected by rapid laboratory tests. Sometimes the characteristic virus symptoms can be identified in the field on the base of symptoms they produced, however there are some virus-like symptoms, that can be induced by the other reasons. Besides, on the infected grapevines no external symptoms of disease may be found. Such latent infection can be detected only during the laboratory testing. The aim of this research was testing the grapevine seedlings imported to Ukraine from different countries.

MATERIALS AND METHODS

For detection of grapevine latent infection by GFLV and GLRaV-1-9 one seedlings was selected from one hundred plants

Viral infections were confirmed by ELISA and RT-PCR. For ELISA test-systems produced by Agritest (Italy) was used. For RT-PCR total RNA was extracted from 0,1 g fresh bark scrapings. The RT-PCR was realized according Rowhani procedure [*Rowhani A. et al.*, 1993].

The RT reaction was heated at the thermostat during 30 min at 52C followed by 35 cycles of PCR amplification using primers CPV and CPC (GLRaV-1), With 547 and N of 229 (GLRaV-3), oligoc1 and oligov1 (GFLV) [*MacKenzie D. J. et al., 1997*] and the profile: 30 s at 94 ◦ C, 45 s at 56 C and 60 s at 72 ◦ C . The elongation time at the last cycle reached 7 min. During our research the annealing temperature was 52C for GLRaV 1 and 60C for GFLV. Reaction mixture of total volume 25 mkl contained 2 mM dNTP mix, 0,1 M DTT, 10 pM specific viral primers and 1,25 U of Taq DNA polymerase (AmpliSens, Russia), 8 U of reverse transcriptase (AmpliSense, Russia) and 1,5 mM $MgSO_4$ [*Rowhani A. et al.*, 1993].

The reaction was conducted at programmed thermostat "Rotor-Gene 6000" (Corbett, Australia).

RESULTS AND DISCUSSION

It was established that latent infection by GFLV and GLRaV 1-3 was found at the planting material from Moldova and Slovenia (Tabl. 1 and 2, fig. 1)

Table 1. GLRaV 1-3 detection in the seedlings introduced to Ukraine

Genetic origin	Country			
	Moldova	Slovenia	Germany	France
Cabernet Sauvignon	+	+	-	-
Chardonnay	+	-	-	-

Table 2. GFLV detection in the seedlings introduced in Ukraine.

Genetic origin	Country			
	Moldova	Slovenia	Germany	France
Cabernet Sauvignon	+	-	+	-
Chardonnay	+	-	-	-

The GFLV was found in the seedlings introduced to Ukraine from Moldova and Germany. The testing of imported planting material in the laboratory helped to prevent the distribution of infected planting material in Ukraine.

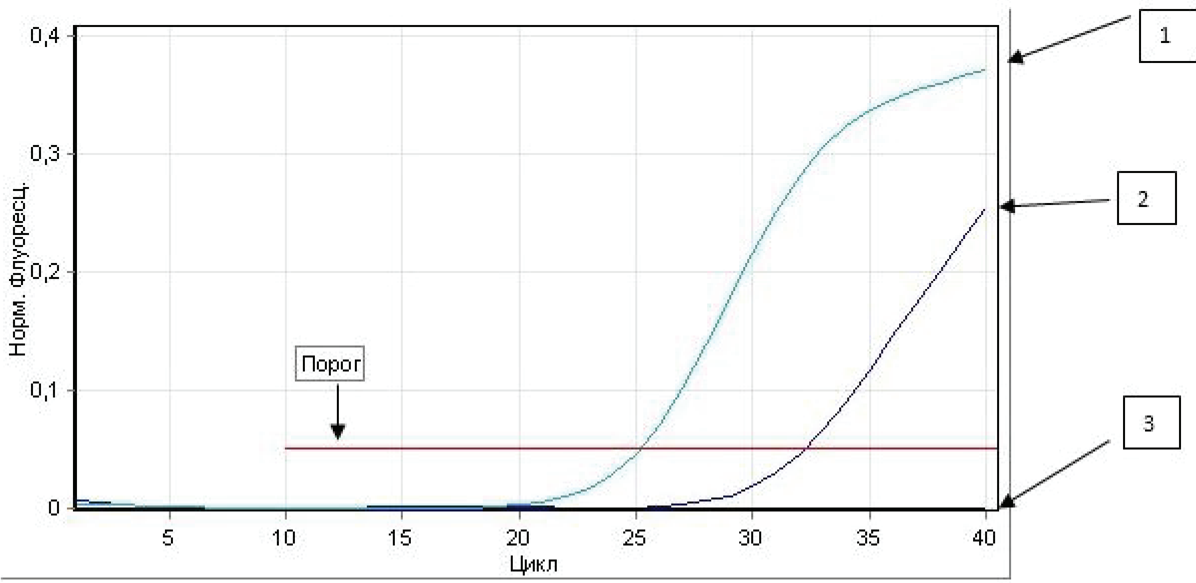

Figure 1. Detection of the GFLV grapes by RT-PCR in real time. 1- sample is infected with a GFLV; 2 - positive control, 3 - negative control.

It was established that the grapevine planting material from from Moldova was latently infected both GFLV and GLRaV-1-3. The laboratory analysis by ELISA and RT-PCR help us to detect latent infection by GLRaV and GFLV and prevent the distribution of infected plants.

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PP 03 - Molecular evidence of ArMV and related satellite RNA in Portugal

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INTRODUCTION

Together with Grapevine fanleaf virus (GFLV), Arabis mosais virus (ArMV) is implicated in grapevine infectious degeneration disease (GID), a complex of diseases affecting grapevine worldwide (Andret-Link *et al*., 2004). Symptoms associated with GID range from malformation in leaves and canes to severe leaf chlorosis.

The two Nepoviruses are RNA-containing viruses with a genome consisting of two single-stranded positive sense RNAs, called RNA-1 and RNA-2. Both RNA1 and RNA2, which 122 kDa polyprotein comprises the capsid protein, are required for infection (Wetzel *et al*., 2001; Wetzel *et al*., 2004). For ArMV, genome sequences of only a few isolates have been fully determined.

Some GFLV and ArMV isolates have been shown to support the replication of large satellite RNAs (satRNA), which are dependent on the helper virus genome for replication, encapsidation and systemic spread (Pink *et al*., 1988; Liu *et al*., 1990; Gottula *et al*., 2013).

ArMV and Grapevine fanleaf virus (GFLV) are serologically distant related viruses (Martelli *et al*., 1993). Routine detection is usually done with DAS-ELISA using commercial antibodies specific of each virus.

Screening for both viruses with the available serological tools has indicated the presence in Portugal of isolates testing positive for ArMV. However, these infections could not be verified by molecular assays using RT-PCR developed by other authors, so information is lacking on the causative ArMV variants. The immediate objective of this study was to ascertain the presence of ArMV in Portugal through molecular evidence, as background to implementing an effective detection protocol.

MATERIALS AND METHODS

Plant material and virus source

The isolates analyzed in this work were each obtained from samples screened by the INIAV plant protection services. All isolates analyzed had been found positive for ArMV using commercial antibodies.

RNA extraction

Total plant RNA was extracted from each isolate with an E.Z.N.A.TM Plant RNA Kit (Omega Bio-Tek, USA), after leaf tissue was ground in liquid nitrogen and homogenized in extraction buffer (4 M guanidine thiocyanate). The kit manufacturer's instructions were followed for the remaining extraction steps.

cDNA synthesis and PCR

Synthesis of cDNA using a iScript cDNA First Strand Synthesis Kit™ (Bio-Rad, USA) was done according to the manufacturer's protocol in a final volume of 20 μl, with 5 μl of total RNA.

PCR reactions were performed in a final volume of 50 μl with Pfu DNA polymerase (Fermentas, Thermo Fisher Scientific, Inc.). The primer pairs tested in this work were retrieved from the literature pertaining to the detection and sequencing of ArMV variants and amplification of satRNA.

Cloning and sequencing

The amplicons obtained for each isolate, with different primer pairs, were ligated with the CloneJET™ PCR Cloning Kit (Fermentas, Thermo Fisher Scientific, Inc.) and used to transform *E. coli* XL1Blue (Agilent Technologies Inc., USA) competent cells. The PCR products of at least 16 positive clones per isolate were next analyzed by single-strand conformation polymorphism (SSCP). For each SSCP pattern detected, the respective recombinant clones (purified plasmid with insert) were obtained using an E.Z.N.A. Plasmid Miniprep Kit II (Omega Bio-Tek, Inc.), prior to commercial sequencing (Stab Vida,

Caparica, Portugal).

Sequence data analysis

The sequences obtained for each isolate under study were visualized and aligned with homologous sequences retrieved from GenBank using BioEdit Sequence Alignment Editor (Bioedit) (Hall, 1999) and ClustalW (Thompson *et al*., 1994). Phylogeny was inferred using the Maximum Likelihood (ML) method implemented in MEGA5 (Tamura *et al*., 2011). Bootstrap values were estimated with 1000 replicates

RESULTS AND DISCUSSION

Partial sequences of the capsid protein of ArMV were obtained for only one isolate (Cabernet Sauvignon, CS30404) of the set found positive in DAS-ELISA, and only with one of the primer pairs tested. Satellite RNA sequences were also retrieved from this isolate. The preliminary results of the molecular evidence gathered are shown in Figs. 1 and 2.

The difficulty in detecting ArMV through RT-PCR might indicate that divergent variants are present in the Portuguese isolates. Further work is underway to fully characterize the ArMV isolates so far detected.

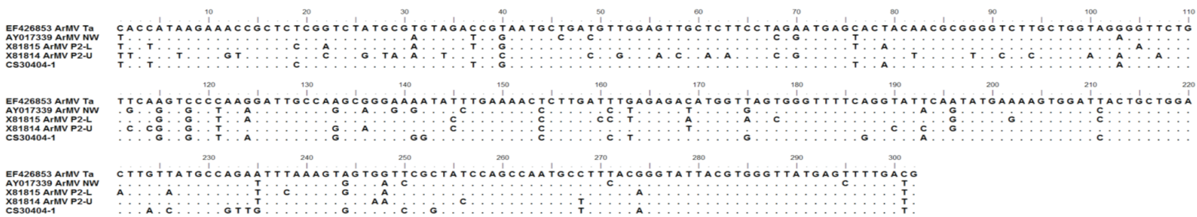

Figure 1. Alignment of partial sequences of the capsid protein of ArMV. Sequences retrieved from GenBank are indicated by accession number and isolate name.

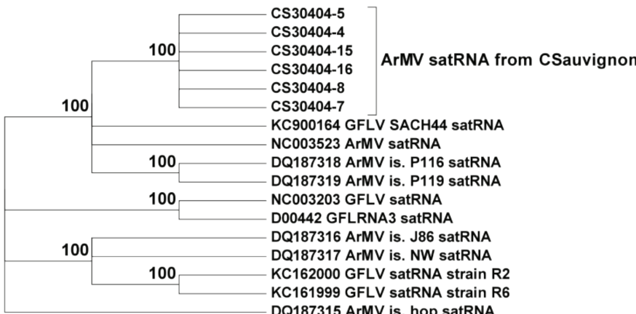

Figure 2. Phylogenetic analysis of satRNA sequences retrieved from ArMV and GFLV isolates The dendrogram was constructed by the maximum-likelihood (ML) method. Bootstrap values of 1000 replicates are shown at the nodes and branches reproduced in less than 95 % of bootstrap replicates are collapsed. Sequences retrieved from GenBank are indicated by accession number and virus name.

ACKNOWLEDGMENTS

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PP 04 - Serological, biological characterizations of some Grapevine viruses and the epidemiological spread of the *Nepovirus* in Syria

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INTRODUCTION

The vine (*Vitis Vinifera* L.) is one of the most important fruit trees planted in Syria. Among them, at least 37 viruses can naturally infect vine tree. *Grapevine leafroll-associated virus 1* (GLRaV-1), *Grapevine leafroll-associated virus 3* (GLRaV-3), *Grapevine leafroll-associated virus* 7 (GLRaV-7) (genus *Ampelovirus*, family *Closteroviridae*) have been the most dangerous virus in Syria. Problems were mainly ascribed to the soilborne virus; *Nepovirus*; *Grapevine fanleaf virus* (GFLV) (genus *Nepovirus*, family *Comoviridae*), the insect transmitted GLRVs and other grapevine viruses like *Grapevine virus A* (GVA) (genus *Vitivirus*, family *Flexiviridae*) and *Grapevine fleck virus* (GFkV) (genus *Maculavirus*, family *Tymoviridae*).

OBJECTIVES

1) Serological characterization of six grapevine viruses (GFLV, GLRV 1, 3 and 7, GVA and GFkV) in the western heights of Syria. 2) Biological characterization of (GLR-1). 3) Epidemiological spread of the *Nepovirus*-GFLV.

RESULTS

A total of 64 samples were collected and analysed in the laboratory by DAS-ELISA. Of these, 55 (85.93%) (Table 1) were found to be infected via GLRV-1 (48/64), GLRV-3 (44/64), GLRV-7 (36/64), GFLV (43/64), GVA (3/64) and GFkV (5/64) (Table 2). GLRVs were detected in leaf-rolled plants from all regions visited and the viral diseases was very high in Tartous then RiF-Dimashiq and Qunitera. Also, Qunitera had the highest incidence of GFLV. Whereas, GFkV was found in all samples excepted Tartous and Rif-Dimashiq samples. Samples from Lattakia, Homs and Rif-Dimashiq provinces were tested for the absent of GVA which found only in Tartous and Qunitera. Moreover, different mixed virus infections were observed in some of the samples collected in almost grapevine samples. The *Carbenet France* indicator plant inoculated with infected samples (GLR-1) via grafting way, developed leaf-reddening symptoms by 3 to 5 months after inoculation. Symptoms were very virulence on the grapevine cultivar *Kober 5BB.*

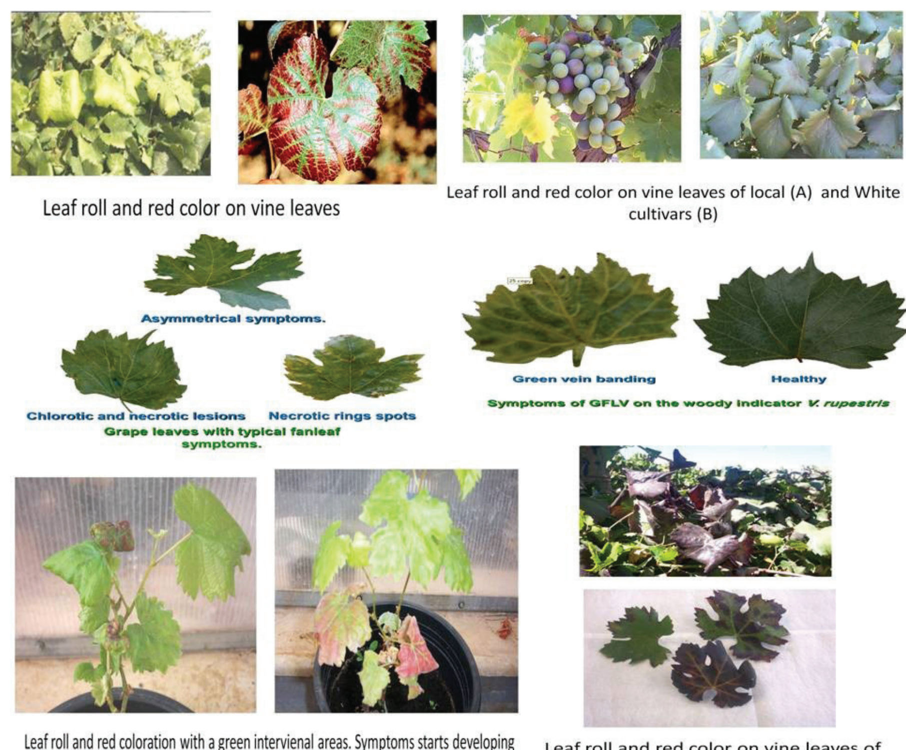

Table 1. Number of grapevine samples which showed symptoms suggestive of virus infection, collected from different fields during 2009 in Syria.

Province	N. of infected samples	Percentage of virus infection (%)
Lattakia	11/14	78.57
Tartous	14/15	93.33
Homs	4/5	80
Rif-Dimashiq	11/12	91.66
Qunitera	15/18	83.33
Total	55/64	85.93

Table 2. Results of serological tests conducted on grapevine samples which showed symptoms suggestive of virus infection, collected from different fields during 2009 in Syria.

Province	N. of samples tested	N. of samples positively reacted with antisera to					
		GLRV-1	GLRV-3	GLRV-7	GFLV	GVA	GFkV
Lattakia	11/14	11	9	5	9	0	1
Tartous	14/15	14	14	11	14	1	0
Homs	4/5	3	3	1	3	0	1
Rif-Dimashiq	11/12	5	6	4	2	0	0
Qunitera	15/18	15	12	15	15	2	3
Total	55/64	48/64	44/64	36/64	43/64	3/64	5/64

The epidemiology and Spreading of GFLV in Syria. GFLV was detected in all infected parts of plant and soil from all selected sites visited in Qunitera (Table 3). GFLV existed in roots, bark, leaves via very high infection in January and February during 2010. But it occurred in pre-blossoming clusters just in May 2009. This is first record of epidemiology and spread of GFLV in Syria.

Table 3. Results of serological tests conducted on grapevine fanleaf virus (GFLV) which collected from different parts of grapevine plant in Qunitera, Syria during 2009-2010.

Month	Soil	Roots	Bark	Leaves	Pre-blossoming clusters	Fruit clusters
April 2009	+	+	+	+++	-	-
May	+	+	+	+++++	++++	-
June	++	++	++	+++++	-	-
July	++	++	++	+++	-	-
August	++	++	++	++	-	-
September	++	++	++	+	-	-
October	++	++	++	+	-	-
November	++	++	++	-	-	-
December	+++	+++	+++	-	-	-
Jan. 2010	+++	+++	++++	-	-	-
Feb.	+++	+++	++++	-	-	-
March	++	++	++	-	-	-

+++++: very high absorbance, + : very low absorbence, - : not tested parts

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PP 05 - Occurrence of Grapevine Leafroll Associated Virus in Algerian vineyards

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INTRODUCTION

Viticulture in Algeria is experiencing a relatively steady development over the last year with area increased from 50 000 ha in 1993 to 68,564 in 2013 ha. yeld were consistently increased from 20qt/ha to 80qt/ha . However, this renew of viticulture is confronted to the spread of several diseases that threatens this culture. Our work has focused on the study of one of the most common grapevine virus diseases in the world, namely the grapevine leafroll disease.

MATERIALS AND METHODS

The field study and sample collection were conducted on autumn 2010 and 2012 in table and wine varieties in western (Aïn Témouchent and Mascara) and central (Algiers, Tizi-Ouzou and Boumerdes) region of Algeria. A total 584 samples were collected from individual vines in different varieties (30 varieties) including commercials and grapevine germplasm collection of Mascara. Mature canes were randomly collected and stored at 4°C.

All collected samples were tested by DAS-ELISA (Clark and Adams, 1977) for the presence of GLRaV-2,-3, -5 and -6 using specific commercial polyclonal antiserum (Agritest, Bari, Italy). The extracts were obtained by macerating leaf tissues in the PBS-buffer. Absorbance was recorded at 405 nm using an automatic microplate reader (Multiskan Ascent, Labsystems, Waltham, MA, USA). Samples with absorbance readings exceeding or equal three times that of the healthy samples were considered positive.

Grapevine leafroll associated virus -2, -3 and -5 were investigated by RT-PCR using specific primers (tab. 1).

Table. 1: Primer used in RT-PCR

Virus	Primer	Sequence	Product size (bp)
GLRaV-2	L2-F	ATAATTCGGCGTACATCCCCACTT	331
	U2-R	GCCCTCCGCGCAACTAATGACAG	
GLRaV-3	LC1F	CGCTAGGGCTGTGGAAGTATT	546
	LC2R	GTTGTCCCGGGTACCAGATAT	
GLRaV-5	LR5-1F	CCCGTGATACAAGGTAGGACA	690
	LR5-1R	CAGACTTCACCTCCTGTTAC	

RESULTS AND DISCUSSION

To highlight the importance of Grapevine Leafroll Disease, a study was conducted in the eastern and western vineyards of Algeria. The surveys reveal the presence of leaf roll symptoms in the majority of the prospected vineyards. To highlight the importance of this disease, 584 samples were collected and ELISA test was performed against GLRaV-2, -3, -5 and -6. The results showed the presence of all viruses analyzed with a total percent infection of 55.7% (325 of 584 samples). The results showed also the predominance of GLRaV-3 compared to the other viruses with 47.8% followed by the GLRaV-2 with 15.8%. Lekikot et al. (2012) reported 55,3% and 8,1% respectively for GLRaV-3 and -2. Six samples were found positive for GLRaV-5 and only two for GLRaV-6, this results may be considered as the first report of this two variants of GLRaV-4 from Algeria by ELISA test.

The high prevalence of GLD in Algeria may be due to the use of infected material and the presence of its vector. From this study, we reveal the high infection of grapevine germplasm collection for autochthonous varieties.

Symptoms of leafroll were observed on several mother blocs. A more detailed study on the health status of mother blocs is needed to achieve a more efficient health selection program. Some vineyards surveyed in 2010 were torn off and replaced due to the economic losses caused by the decline of yield and grape quality.

Positive samples for the GLRaV-2, -3, and -5 were tested by RT-PCR and sequencing of part of the HSP70h and Cp gene was performed for all three viruses. BLAST search confirm these sequences and they were submited to European Nucleotide Archive (ENA).

	sample tested	total infection (%)	LR2(%)	LR3 (%)	LR5(%)	LR6(%)
Dattier de Beyrouth	91	56	18,7	49,5	2[a]	
Gros noir	100	66	29	60		
Cardinal	62	50	6,5	50		
Alicante Bouchet	61	62,3	24,6	44,3	1[a]	1[a]
King's Rubi	9	88,9	22,2	88,9		
Muscat	70	50	10	37,1		
Merseguerra	10	70	0,0	70		
Cinsault	40	15	7,5	7,5		
Carignan	31	32,3	9,7	32,3		
Valensi	62	48,4	14,5	32,3	2[a]	1[a]
Chasselas	9	100	0,0	100		
Chaouch blanc	10	100	10	100	1[a]	
Autochtones	29	82,8	6,9	79,3		
total	584	55,7	15,8	47,8		

*Out of 100 sample

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PP 06 - Characterising the suppressors of silencing encoded by *Grapevine leafroll-associated virus 3* and their activity in New Zealand genetic variants

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INTRODUCTION

Grapevine leafroll-associated virus 3 (GLRaV-3) infects grapes, is present in all grapevine growing regions worldwide and can result in uneven bunch ripeness and low quality wine. This virus threatens the largest single horticultural export earner for New Zealand, the grape and wine industry. One potential method to prevent the impact of GLRaV-3 is through cross protection, where a virus strain that produces only mild symptoms protects against infection from a more severe strain of the same virus (Ziebell and Carr, 2010). However, a mild strain of GLRaV-3 has not been identified yet.

Viruses encode suppressors of silencing (VSRs) to bypass the host plant's RNA interference defence system. VSRs can act either locally or systemically and some VSRs possess both local and systemic VSR activity e.g. the p20 kDa protein encoded by *Citrus tisteza virus* (CTV) (Lu et al., 2004). We aim to characterise the VSRs encoded by GLRaV-3 and to understand the impact of the genetic variation observed in the genetic variants present in New Zealand. This knowledge can then be used to isolate and/or generate mild strains of GLRaV-3 for use in cross protection.

Prior to this research, the only described VSR encoded by GLRaV-3 was the p19.7 kDa protein (encoded by ORF10) which was shown to act locally (Gouveia et al., 2012). The sequence of the p19.7 kDa protein varies greatly among the GLRaV-3 isolates across the different phylogenetic groups, particularly with the two novel New Zealand isolates that have high amino acid divergence from other GLRaV-3 sequences (Group VI v. Group I are 38% divergent, and NZ2 v. Group I are 36% divergent). Therefore, we initially focused on the activity of this p19.7 kDa protein. We used both different methods to investigate the local and systemic VSR activity of the p19.7 kDa protein from seven described GLRaV-3, Groups I-VI and NZ2.

MATERIALS AND METHODS

The ORF10 of GLRaV-3 genetic variants representative of Groups I to VI and NZ2 were cloned into pHEX2 (Hellens et al., 2005) to form a set of expression clones that were then transformed into *Agrobacterium tumifaciens* strain GV3101. The relative luminescence values were measured for luciferase and renilla after 4-5 days using the dual luciferase reporter (DLR) assay system (Promega, Madison, WI, USA) as described by the manufacturer. Infiltrations were performed on 4 week old *Nicotiana benthamiana* plants (eight replicates per experimental treatment).

For local, and subsequent systemic VSR GFP assays, *A. tumefaciens* grown to OD_{600} 0.6 was infiltrated as above. Each *N. benthamiana* 16C plant was infiltrated once and five plants were infiltrated for each treatment. GFP fluorescence was assessed 7 dpi for local VSR activity and for systemic VSR activity newly emerged leaves at the top of each plant were assessed at 15 dpi.

RESULTS AND DISCUSSION

The DLR assay showed that the p19.7 kDa protein encoded by the Group III isolate of GLRaV-3 had the strongest local VSR activity which, along with Group I encoded p19.7 kDa protein, was even stronger than that observed from the p19 VSR encoded by *Tomato bushy stunt virus* (TBSV) (Figure 1). The NZ2 isolate encoded the p19.7 kDa protein with the weakest local VSR activity. Groups II, V and VI isolates of GLRaV-3 encoded p19.7 kDa protein with similar strengths of local VSR activity that were stronger than that of the p19.7 kDa protein encoded by the Group IV isolate. Infiltration of the GFP construct along with any of the seven GLRaV-3 p19.7 kDa protein constructs resulted in green fluorescence indicative of local VSR activity (data not shown), and validation of the DLR assay results.

Using the CTV p20 kDa protein as a comparison, we investigated the possible systemic activity of the p19.7 kDa proteins encoded by the isolates from each of the seven GLRaV-3 taxonomic groups (Figure 2). The systemic VSR activity assay

showed that the p19.7 kDa proteins encoded by the GLRaV-3 Group I and NZ2 isolates have no systemic activity, as only red auto-fluorescence was observed. By contrast, the GLRaV-3 p19.7 kDa proteins encoded by Groups II to VI all exhibited systemic VSR activity, as demonstrated by the green fluorescence in newly grown tissues.

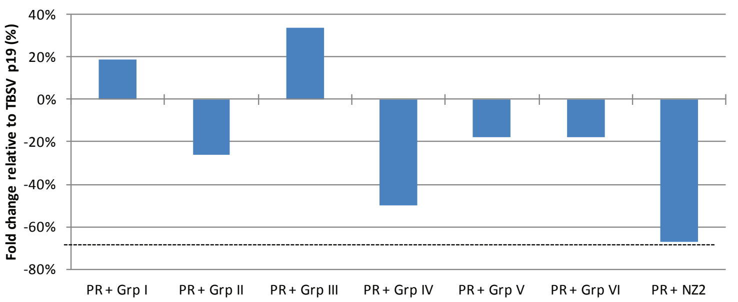

Figure 1. Proportion of local VSR activity relative to TBSV encoded p19.

The ratio of luciferase and renilla luminescence was compared for each of the seven GLRaV-3 isolate p19.7 kDa proteins tested with the PR construct. Data is presented as a percentage fold change in local VSR activity relative to TBSV p19. Dotted line represents the ratio observed with no local VSR activity.

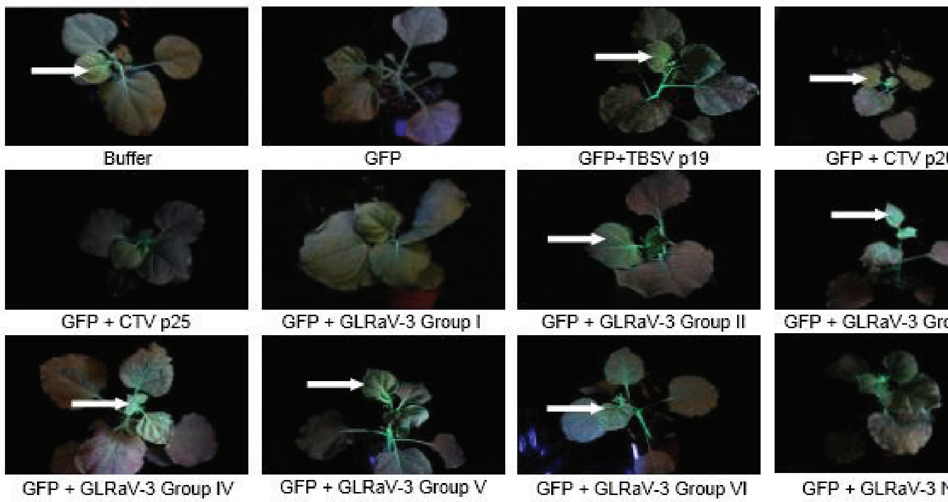

Figure 2. Systemic GFP assay for virus encoded suppressor of silencing activity. Images of *N. benthamiana* 16C plants at 15 days following infiltration with *A. tumefaciens* carrying vectors encoding the named proteins. White arrows indicate green fluorescence in 16C plants infiltrated with buffer or VSRs showing systemic activity.

This research has shown preliminary evidence for local VSR activity of the p19.7 kDa proteins encoded by Group VI and NZ2 isolates of GLRaV-3, the first demonstration of systemic VSR activity from a GLRaV-3 encoded protein (p19.7 kDa protein encoded by Group II - VI isolates of GLRaV-3), and the validation of the Dual Luciferase Renilla assay for rapid identification of local VSR activity.

Research showing the molecular evidence for these activities and the relative strength of local and systemic activity will validate these preliminary findings. In addition, experimentation on additional GLRaV-3 genes will determine the total number and type of VSRs encoded by GLRaV-3 and its molecular variants. This project now sets the scene for future research on the VSR activity encoded by GLRaV-3 and use of this knowledge in developing mild strains and infectious clones of GLRaV-3.

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PP 07 - Grapevine Leafroll associated virus 3: Effects on rootstocks, vine performance, yield and berries

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INTRODUCTION

Grapevine leafroll disease is one of the most important viral diseases affecting grapevines worldwide. The disease causes the yield losses of as much as14-40% with delay of 3 weeks to a month in fruit maturation (Rayapati et al., 2014). Grapevine leafroll associated virus 3 (GLRaV-3) is the type species of the genus *Ampelovirus* in *Closteroviridae* family and is considered as the most important causative agent of grapevine leafroll disease. The virus transmits in the field by number of different mealybugs and scale insects (Tsai et al., 2010).

Our past research has shown that the effects of infection by the GLRaVs depend greatly on the virus as well as the grapevine variety and the rootstocks. In our research, Cabernet franc vines budded onto nine different rootstocks were inoculated with GLRaV-3 from three different sources and planted in the field to evaluate the symptoms, plant growth, yield, berry qualities and berry compositions.

MATERIALS AND METHODS

Reference sources of leafroll viruses were established in the Davis Grapevine Collection (Golino, 1992) and regularly updated with newly found viruses and virus strains. Three different isolates of GLRaV-3, LR101, LR127 and PA94.148, used in this experiment were from this collection. Cabernet franc plants were propagated on the following 9 rootstocks: AXR1 (*V. vinifera* 'Aramon' X *V. rupestris* 'Ganzin'), Mgt 101-14 (*V. riperia* X *V. rupestris*), 110R (*V. berlandieri* X *V. rupestris*), 3309C (*V. riparia* X *V. rupestris*), Kober 5BB (*V. berlandieri* X *V. riparia*), 420A Mgt (*V. berlandieri* X *V.riparia*), Freedom (1613C OP seedling X Dog Ridge OP seedling); St. George 15 (*V. Rupestris*) infected with *Grapevine rupestris stem pitting associated virus* (GRSPaV) and St. George 18 free from GRSPaV. In 2009 the rootstock portion of these plants was inoculated with two chip buds from each virus source. The inoculated plants were planted in the field which included 15 replicate per treatment per rootstock in three different blocks (5 replicate per block). Symptoms, vine growth (cane length, pruning weight, berry weight, total clusters, and total yield) and berry composition (percent moisture, anthocanins, potassium, malic acid, ammonia, NOPA, pH, brix, titratable acidity, tartaric acid, and YAN) were evaluated in 2014. The symptoms were rated in October from 0 to 4 where 0 represented vines with no symptoms and 4 for vines showing very severe symptoms. For berry composition evaluation, the fruits were harvested when the Brix was approximately 24%. Cases where treatments showed significant differences among rootstocks, leading to significant virus x rootstock interactions, were analyzed and reported by rootstock. JMP® software (version Pro 11, SAS Institute, Inc., Cary, NC) was used for two-way analysis of variance and Tukey's honestly significant difference test at the $p \leq 0.05$ significance level was used to separate means of different treatments.

RESULTS AND DISCUSSION

The real time RT-PCR test results showed that Isolate LR101 was co-infected with *Grapevine virus A* (GVA) and PA94.148 was co-infected with GRSPaV.

In general, the symptoms rating on the majority of the plants inoculated with any of the three isolates of GLRaV-3 was 3-4 which indicated quite severe. Only the symptoms produced on Cabernet franc propagated on AXR rootstocks were milder and rated 2-3.

Statistical analyses indicated that there was no virus x rootstock interaction for cane length, berry weight, total clusters, and total yield. Therefore, virus main effects were determined across all rootstocks. Cane length was significantly less for all three isolates compared to healthy while berry weight, total clusters and total yield were not significantly different from healthy. The virus effect on pruning weight depended on both the isolate and the rootstock. In AXR.1 vines, all three isolates significantly reduced pruning weight compared to healthy. Conversely, there were no significant virus effect on pruning weight in 101.14, 110R, 420A, and Freedom vines. The virus effect on pruning weight in 3309C, 5BB, St. George 15, and St. George 18 vines was variable but was always significant for at least one isolate.

The effect of virus treatments on berry composition was highly variable among isolates and depended on rootstock as indicated by a significant virus x rootstock interaction. Percent moisture was the parameter most affected by all three isolates across all rootstocks; only 420A and St. George 15 showed no significant virus effects. Conversely, tartaric acid was the least affected parameter with the only significant difference occurring on LR101-infected vines grafted on 110R. Significant virus effects on brix and titratable acidity occurred for all three isolates on 5BB but were either absent or limited to one or two of the isolates on the remaining eight rootstocks. The virus effect on anthocyanin, potassium, malic acid, ammonia, NOPA, pH, and YAN was variable, and depended on the isolate and the rootstock.

With regard to rootstocks, significant virus effects across all parameters were highest on 5BB, followed by 3309C, Freedom, 110R, St. George 18, St. George 15, AXR.1, 101.14 and 420A..

In conclusion, the results suggest that GLRaV-3 isolates LR101, LR127 and PA94.148 do affect vine performance and berry composition but for most parameters, the effect is variable among isolates and in the case of pruning weight and berry composition, also depends on the rootstock.

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PP 08 - Current status of grapevine leafroll associated viruses in East Mediterranean Region of Turkey

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INTRODUCTION

Turkey is one of the main grape producers contry in the world. According to data of FAO, Turkey has 504.000 ha of grapevine cultivation area and comes from after Spain, France, Italy and Chine at 5th level. Also, annual grape production is approximately 4.300.000 tons, the production level is at 5th after same cuntry.The research was conducted East Mediterranean Region where grapevine is intensively cultivated. Major growing provinces of this region are Adana, Mersin, Gaziantep, Kilis and Hatay. Leafroll is one of the most widespread virus diseases and present in all grape growing regions of the world. Disease affects the development and quality of grapes, delays ripening and depresses bery sugar content resulting in reduced wine quality. It may cause up to 40% yield reduction of grapes. Leafroll viruses existed in the Mediterranean *Vitis vinifera* and in the American *Vitis* species. Because of the symptomless on American rootstock and *Vitis* species, the disease is spread very easly and quickly. The major virus and virus-like diseases of grapevine are previously reported in Turkey (Martelli,1987; Caglayan, 1997; Cigsar et al, 2002; Buzkan et al, 2009; Turkmen et al, 2012, Kaya et al, 2012). The aim of this study was determine the status of the diseases in recent years in East Meditarranen Region.

MATERIALS AND METHODS

Surveys were conducted in commercial vineyards of East Mediterranean Region in 2012 and 2013. 138 leaf samples were collected. Each vineyards were checked for typical symptoms of leafroll virus diseases. Such as foliar discolarations expand and coalesce to form a redish-purple color within the interveinal areas of the leaf, in red-fruited cultivar consist of green veins and reddish interveinal areas, roll downward of leaf, whole redness of leaf. Most of samples which collected from vineyards have this type of symptoms.

Samples were analyzed by DAS-ELISA (Clark and Adams, 1977) using commercial diagnostic kits obtained from BIOREBA AG (Switzerland). Using kits include GLRaV-1, GLRaV-2, GLRaV-3, GLRaV-4-9, GLRaV-6, GLRaV-7. Test were conducted according to the company's proposed.

RESULTS AND DISCUSSION

Totally 138 symptomatic samples were randomly collected and tested. Some of samples were found single infection, most of them were found mix infection (2-3). Unlike other region, the most widespread infection was GLRaV-1 which present 96 number in total. Then followed by 53 samples GLRaV-4+9, 45 samples GLRaV-3, 19 samples GLRaV-2 and only 2 samples were found positive infection for GLRaV-6. This is the first report on the presence of GLRaV-6 in East Mediterranean Region.

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PP 09 - Current status of Grapevine leafroll associated viruses in Hatay and Gaziantep provinces in Turkey

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INTRODUCTION

Turkey is one of the world's leading grapevine production countries with the total production of 4.175.356 tons in 2014 (Anonymous, 2015). Grapevine leafroll disease (GLD) is one of the most economically important viral diseases. GLD is associated with a complex of filamentous viruses referred to as Grapevine leafroll-associated viruses (GLRaVs). All GLRaVs identified so far belong to the family *Closteroviridae*. Up to now, 11 different GLRaVs have been identified:one in the genus *Closterovirus* (GLRaV-2), nine in the genus *Ampelovirus* and one in the new-defined genus *Velarivirus* (GLRaV-7) (Martelli et al., 2012). Some of the viruses among this family have already been detected in Thrace, Aegean, Central and Southeast regions of Turkey by using biological and serological methods. Molecular detection tools have recently been used in our country for grapevine viruses and except a few studies, the sanitary status in Turkey has been investigated by the conventional diagnostic tools (Buzkan et al. 2010; Kaya et al. 2012). Therefore, we have undertaken a survey of *Grapevine leafroll associated virus 1, 2, 3, 4, 5, 6, 7, 9* (GLRaV-1, -2, -3, -4, -5, -6, -7, -9) in Hatay and Gaziantep provinces and samples were tested both by ELISA and RT-PCR. The aim of the present study was to evaluate in commercial vineyards the occurrence of these viruses and their incidence rate. The results will help to evaluate the economic impact of viral diseases and to define an efficient control strategy to mitigate their negative impact.

MATERIALS AND METHODS

Eleven vineyards in Hatay and 2 vineyards in Gaziantep were selected on a random basis. Totally, 153 samples were collected in January 2013. To account for the possible uneven distribution of the viruses within a grapevine, four dormant canes per plant were collected and bulked for virus testing. All samples were tested by double antibody sandwich ELISA (DAS-ELISA) by using the commercial kits from BIOREBA (Reinach, Switzerland) for *Grapevine leafroll-associated virus* (GLRaV) -1, -2, -3, -4-9, -5, -6, -7. All ELISA positive samples were confirmed by RT-PCR analysis using already published primer pairs (Osman and Rowhani 2005; Komorowska et al. 2014).

RESULTS AND DISCUSSION

Samples from 153 individual grapevines were collected from 13 vineyards. The viruses GLRaV-1, -4-9, -2 and -3 were predominant in Hatay and Gaziantep vineyards. Grapevine leafroll disease appeared to be widespread in both provinces as nearly half of the locations were infected by GLRaV-1 or GLRaV-4-9. According to the DAS-ELISA results, the most common viruses were GLRaV-1 (55.56%), GLRaV 4-9 (43.14%), GLRaV-2 (15.69%) and GLRaV-3 (12.42%). GLRaV-6 was occasionally detected (0.65%) and all tested samples were negative for GLRaV 5 and -7 (Table 1). It was already detected that mixed infection of leafroll associated viruses are quite common in infected vines (Agran et al. 1990 and Gomez Talquenca et al. 2003). The most widespread virus, GLRaV-1 was generally found in this study in vines infected with GLRaV-4-9 (25.49%) and -2 (3.27%). In addition, triple combination of GLRaV-1 was detected with GLRaV-2 and -4-9 (7.84%) and also -3 and -4-9 (3.92%). ELISA results were confirmed by RT-PCR analysis. As reported by various authors in Turkey, GLRaV-1 and GLRaV-3 were the most common viruses as single or mixed infections in different regions (Köklü et al., 1998; Sarpkaya et al., 2004). GLRaV-5 and -7 were not detected in this study both by ELISA and RT-PCR whereas Akbaş et al. (2007) and Buzkan et al. (2010) reported GLRaV-7 in Central Anatolia and -5 in South eastern Anatolia with a very low infection rate, respectively. It could be due to different sampling method, number of collecting samples and also cultivars. This study conclusively demonstrates the last status of leafroll infection of grapevines in two important grapevine growing provinces in Turkey.

Table 1. Infection rates of Grapevine leafroll associated viruses in grapevines growing in Hatay and Gaziantep provinces by DAS-ELISA

Viruses	NIS / NTS*	Infection rate (%)
GLRaV-1	85/153	55.56
GLRaV-2	24/153	15.69
GLRaV-3	19/153	12.42
GLRaV-5	0/153	0.00
GLRaV- 4,9	66/153	43.14
GLRaV-6	1/153	0.65
GLRaV-7	0/153	0.00

*Number of infected samples (NIS)/Number of tested samples (NTS)

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PP 10 - Grapevine leaf roll associated virus infections in Turkey

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INTRODUCTION

Turkey is located in the subtropic climatic region of theworld and its Northeastern part, the Anatolian peninsula, located between Black sea and Caspian sea regions, includes the location from which many important grapevine varieties originate. In Turkey, grapevine cultivation is present since more than 6000 years, and there is veryrich potential of both wild (*Vitis vinifera ssp. sylvestris*) and cultivated grapevine *(Vitis vinifera ssp. sativa*) germplasms. According to FAO data, Turkey has 540,000 ha of grapevine cultivated area, ranking fourth after Spain, France and Italy, and a grapevine production of 3,923 million tons, ranking sixth after Italy, France, China, USA and Spain (Soylemezoglu et al. 2015). Viticulture provides 24.8% of the total yearly fruit production (TUIK, 2008). The main viticultural region is the Aegean Region with 151,401 ha of vineyard area, that is the 31% of the grapevine cultivated area of the country, followed by Mediterranean, Central Anatolia, Eastern Anatolia, and Marmara regions. Worldwide ,nine viruses have been reported to be associated with diseases of grapevine, alla re floem limited and belongs to family Closteroviridae. This virus family comprises three genera, only *Closterovirus* virus genus contains *Grapevine leaf roll associated virus-2* that is transmitted by mechanical inoculation. The other species belong to *Ampelovirus* genus anda re not mechanically transmissible (Martelli, 2012). Recently, GLRaV-5 was detected by Buzkan et al. (2010) in southeastern part of Turkey.

MATERIALS AND METHODS

Virus survey

The main viticulture production areas in Aegean, Central Anatolia, Eastern and Western Anatolian parts of Turkey were surveyed in the late summer of 2009-2010. Severe reddening, inward curling with greening of major leaves were common on most of the samples collected. Leaf extracts of the samples were subjected to DAS-ELİSA assay fort he persence of GLRaV 1-9 using the kits obtained from Bioreba.

RESULTS AND DISCUSSION

Survey results

143 out of 213 infected grapevine samples were detected as infected with one or more grapevine leaf roll associated virus infection (67,14%). The most widespread infection was GLRaV 4-9 at a rate of 81,12%, and was followed by GLRaV-1 at a rate of 75%, GLRaV-2 at a rate of 62 % , GLRaV-3 at a rate of 50,35%, GLRaV-2 at a rate of 48,95% and finally GLRaV-6 at a rate of 5%. GLRaV 1-3 serotypes were widespread in Marmara region and were detected at 69,23%, 53,85% and 57,69% respectively. The ratio of GLRaV 4 and 9 were the highest in Eastern Anatolian region with the ratio of 100%. GLRaV-7 was predominant ın Eagean region where as GLRaV-6 was quite rare in the same area. Leaf roll associated virus infections were detected as mixed infections, and the widely detected mixture was GLRaV-1, GLRaV-2, GLRaV-3, GLRaV-4-9, and GLRaV-7. The infections were so common on wine grape varieties than our local varieties but locals (Tahannebi, Okuzgozu, Bogazkere)

were also detected as infected by one or mixture of leaf roll infections.

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PP 11 - Field spread of phloem-limited viruses in a Mediterranean Vineyard

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INTRODUCTION

During the last decades the procedures of clonal selection of grapevine clones have been improved and updated protocols have been integrated in the official rules of the European Union, including both genetic and sanitary selection. However a vineyard planted with certified virus-free material is still subject to viral infections if viruses and their natural vectors are present in the area. In the scientific literature different infection rates and virus spread rapidity have been reported (Cabaleiro, 2009), the results being necessarily related to the specific conditions in which they have been obtained.

The present study aimed to acquire information on the possible natural spread of viruses in a vineyard located in a coastal Italian region (Liguria) having generally a mild climate throughout the year, with warm summers and mild winters. This can substantially influence the species and the population levels of potential viral vectors, and consequently the risks of viral disease spread.

MATERIALS AND METHODS

The experimental vineyard is located in Albenga (SV) (North-West Italy). The vineyard was planted in 2002 with vines of several cultivars of *Vitis vinifera* L., with a spacing of 2 m between rows and 1.20 m along the row. All plants derived from meristem culture applied to eradicate the viruses originally present in the mother plants; the sanitation procedure allowed to obtain plants which were micropropagated and repeatedly tested by ELISA to confirm that they were free from the most important grapevine viruses (GFLV; ArMV; GLRaV-1, -2 and -3; GVA, GVB; GFkV). The vines of the 10th row, planted with the white grape cultivar 'Bosco', were again tested for virus infections in 2009, 2013 and 2015 by multiplex Reverse-Transcription Polymerase Chain Reaction (mRT-PCR). In 2013 also several plants of adjacent rows were tested by ELISA. All viral assays were carried out on mature canes collected during winter pruning in January/February. Polyclonal antisera and monoclonal antibodies for serological assays were purchased from Agritest (Valenzano, I), except for GVA detection kit which was from Sediag (Longvic, F). For mRT-PCR, total RNA was extracted from phloem scraped from the mature canes following the protocol of Gambino *et al*. (2008); subsequently the assay was carried out as previously described by Gambino and Gribaudo (2006).

RESULTS AND DISCUSSION

Molecular assays (mRT-PCR) for viral detection performed in 2009, 2013 and 2015 on the 'Bosco' plants of the 10th row revealed that, among the grapevine most important viruses, the GLRaV-3 had a quite rapid spread (Table 1); GVA was also detected in a few plants in mixed infection with GLRaV-3. In 2013 the viral monitoring was broadened to other plants in the vineyard, belonging to the cultivar Bosco as well as to other cultivars, through serological assays. The results of the tests are shown in Figure 1 and confirm the diffusion of GLRaV-3, in some cases in double infection with GVA. No new infection by other viruses associated to leafroll disease was detected up to now.

A similar study, although more prolonged in time, was carried out in Piemonte (region bordering Liguria) a few years ago (Gribaudo *et al*., 2011): in that case, about 25% of plants resulted infected 17 years after the vineyard planting. The spread of GLRaV-3 in the Albenga vineyard appeared more rapid, reaching in a single row a level over 50 % in 13 years. The preferential spread of GLRaV-3 was confirmed as well as, in some cases, the diffusion of GVA in double infection with GLRaV-3.

The hypothesis of disease transmission by slow-moving natural vectors is supported by the frequent spatial distribution of newly infected vines in groups along the row, i.e. according to a adjacent vine pattern (Figure 1). In the Albenga vineyard mealybugs (potential insect vectors of GVA and GLRaVs: La Notte *et al*., 1997; Sforza *et al.* 2003) were present. A wider survey performed in 2009 on mealybug vectors of *Ampelovirus* and *Vitivirus* in Northwestern Italy highlighted that mealybug population levels were high in Liguria (Bertin *et al.,* 2010). The same work provided evidence that infections by GLRaV-1, GLRaV-3 and GVA were common in mealybug field populations.

The present study must be widened in space and time and further investigations, including a study of the mealybug population, are certainly needed before drawing conclusions. Nevertheless the rapid spread of some viruses (mainly

GLRaV-3) may much probably be ascribed to natural vectors, i.e. to mealybugs: the climate of Liguria is mild throughout the year and this can favor the survival and development of those mealybugs which are established mainly in the warm countries (Ben-Dov *et al.*, 2010). Further investigations are planned on the virus diffusion in Ligurian viticultural districts as well as on the presence and role of mealybugs.

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Table 1. Presence of viruses in single or mixed infection, as determined by mRT-PCR assays performed on a single row (10th row, see Figure 1) planted with originally virus-free vines of the cv Bosco.

Year	N° of plants tested	GLRaV-3 infections (%)	GLRaV-3 + GVA infections (%)
2009	74	18.9	0
2013	74	37.8	2.7
2015	86	45.3	7

Figure 1. Spatial distribution of newly infected plants as determined by ELISA (2013) and mRT-PCR assays (2015, 10th row only).

PP 12 - Partial molecular characterization of Grapevine Leafroll-associated Virus-1 from two Slovenian vineyards

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INTRODUCTION

Grapevine leafroll disease is one of the most severe viral diseases of grapevine worldwide caused by Grapevine leafroll-associated viruses (GLRaVs). All serologically diverse viruses implicated in this desease have been assigned to the family Closteroviridae, the only virus family that encodes a 70-kDa heat-shock protein homolog (HSP70h). Currently there are five distinct GLRaVs: *Grapevine leafroll-associated virus 1*, -2, -3 -4 and -7. Of these, GLRaV-1 and GLRaV-3 are the most common and widespread in Europe (Martelli et al., 2012). Routine testing is based on DAS-ELISA for which commercial antibodies are available and RT-PCR based detection is also widely used. GLRaVs have already been found in vineyards in Slovenia but they have never been characterized. In this study, we have showed molecular divergence among natural populations of GLRaV-1 isolates collected from two vineyards from various region of Slovenia, using partial nucleotide sequences of the HSP70h.

MATERIALS AND METHODS

Symptomatic samples were collected during 2011 to 2013 from different grapevine cultivars in two vineyards in Dobravlje (western part of Slovenia) and Semič (eastern part of Slovenia).

All samples were tested for the presence of GLRaV-1 and GLRaV-3 by DAS-ELISA according to the manufacturer's instructions (BIOREBA AG, Switzerland). Infections of all were confirmed by RT-PCR using primer pars LR1 hsp70-417/ LR1 hsp70-737 (Osman et al., 2007) for GLRaV-1 and LC1F/LC2R (Turturo et al., 2005) for GLRaV-3. For each isolate, a portion HSP70h was amplified by RT-PCR. Primer pair LR1-HSP-1-F (Alabi et al., 2011) / LR1hsp70-737 was selected to amplify a 975 bp fragment specific to GLRaV-1. The amplicons were cloned and five to seven independent clones for each amplicon were sequenced in both orientations.

Phylogenetic studies were performed using GLRaV-1 sequences available in the GenBank that corresponded in length to the sequences obtained during this work. The phylogenetic relationships were determined using the maximum-likelihood algorithm (ML) of the MEGA6 (Tamura et al., 2013).

RESULTS AND DISCUSSION

Samples from cultivars Pinot gris, Pinot noir, Zelen and Cabarnet sauvignon from Dobravlje and Žametovka, Laški Rizling, Kraljevina and Modra frankinja from Semič reacted positively against GLRaV-1 and GLRaV-3 in DAS-ELISA.

In Dobravlje, 4.6 % of samples showed mixed infection with GLRaV-1 and GLRaV-3 and 64 % tested positive only for GLRaV-3. There was no single infection with GLRaV-1. In Semič, 28.2 % of samples showed mixed infection with GLRaV-1 and GLRaV-3, 43.6 % tested positive for GLRaV-1 and 12.8 % for GLRaV-3. 14 GLRaV-1 samples were used for further analysis.

Only two Slovenian isolates of GLRaV-1 were identical at nucleotide level and three at amino acid level. Phylogenetic analyses made on the HSP70h of GLRaV-1 separated Slovenian isolates into two groups. Isolates from Dobravlje belong to »Group A« and isolates from Semič belong to »Group E« (Fig 1). The analysis of partial HSP70h nucleotide and amino acid sequences of isolates from Dobravlje showed the maximum identity of 96.2 to 98.8 % and 97.5 to 99.3 %, respectively, with isolate from Czech Republic (AY754930). Isolates from Semič shared the highest nucleotide identity of 92.2 to 98 % with Hungarian isolate (HE794021) and amino acid identity of 94.5 to 98.7 % with Slovakian isolate (AY754944).

Although Grapevine leafroll disease is studied in Slovenia, no contribution to international databases has been made available on the molecular variability of GLRaV isolates of Slovenia. Our results contribute with novel information on the GLRaV population in field grown plants of Slovenian origin. Further studies are in progress to characterize additional Slovenian isolates of GLRaVs.

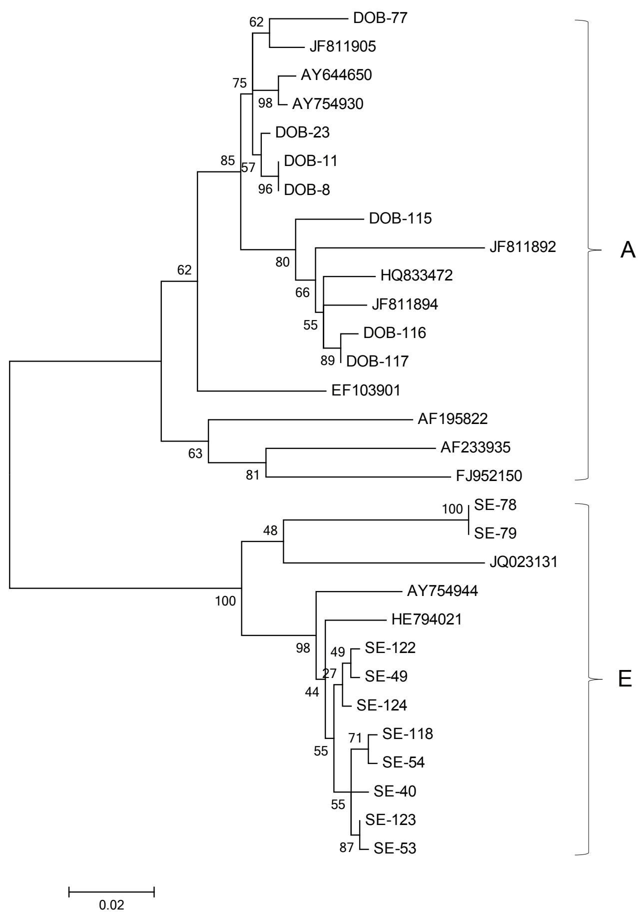

Figure 1. Phylogenetic analyses of GLRaV-1 (ML, K2+G) isolates based on partial nucleotide HSP70h sequences. Three was constructed by the method using MEGA6. Branch lengths are proportional to genetic distances. Bootstrap values (1000 replicates) are given at the branch nodes.

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PP 13 - Genetic variability of *Grapevine leafroll-associated virus 3* (GLRaV-3) from India

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INTRODUCTION

Grapevine is an important commercial horticultural crop of India. Its production is affected by several biotic stresses including viruses. Globally, grapevine is attacked by more than 65 viruses of more than 25 genera and 15 families (Martelli, 2012). Recently grapevine leafroll disease (GLD), an economically dangerous disease, has been found to occur in the vineyards of India (Kumar et al., 2012). GLD is a complex disease caused by eleven viruses belonging to the family *Closteroviridae* and majority of them belonging to the genus *Ampelovirus* of the family *Closteroviridae* (Martelli et al., 2012). Among these, *Grapevine leafroll-associated virus 3* (GLRaV-3) is the most prominent, widely distributed and is the type species of the genus *Ampelovirus* (Martelli et al., 2002). Genetic diversity analysis of plant viruses gives an insight into the biology, ecology and evolution of viruses and together they form the bases for effective management of the concerned diseases (Sharma et al., 2011; Wang et al., 2011). Additionally, a robust knowledge on the diversity of the causal viruses acts as an impetus in proper implication of quarantine and related regulatory measures. Keeping these facts in mind, the eleven isolates of GLRaV-3 were subjected to diversity analysis using a portion of HSP70 h gene and entire p19.7 gene.

MATERIALS AND METHODS

Leaf samples, of ten GLRaV-3 positive grapevines from Nashik and Pune regions of India preserved at -80 °C, were taken for the study. The virus derived from a single grapevine was considered as one isolate (or variant). Samples were subjected to one step reverse transcription-polymerase chain reaction (RT-PCR) following the protocol of Rowhani et al., (2000). Primer pair used to amplify partial HSP70 h were LC1-F —CGCTAGGGCTGTGGAAGTATT and LC2-R —GTTGTCCCGGGTACCAGATAT (Turturo et al., 2005) while the sequences of primer pair specific to complete p19.7 protein (ORF10) are p19.7F - 5' ATGGACCTATCGTTTATTAT 3' and p19.7R - 5' TTTYTAYAGYGCTCCGCAACA 3' (Gouveia et al., 2012). The amplified genomic regions were cloned and sequenced following the standard procedures (Sambrook and Russel, 2001). One previously cloned HSP70h gene from cultivar Cabernet Souvignon was used in HSP70 h study (Kumar et al., 2012). The isolate from Cabernet Souvignon was also included in p19.7 protein gene study. Thus, a total of 11 isolates were selected for the study. The phylogenetic relationship among the isolates studied and GenBank available isolates was inferred using MEGA5 (Tamura et al., 2011). Nucleotide identity matrices were created for both the genomic data set using using *BioEdit* version 7.1.3.0 (Hall, 1999). RDP4 was used for the detection of putative recombination events. Gene- and site-specific selection pressures were estimated employing Datamonkey web-server of the HyPhy package (http://www.datamonkey.org/) (Kosakovsy Pond and Forst, 2005; Kosakovsy Pond et al., 2005).

RESULTS AND DISCUSSION

Phylogenetic study based on partial HSP70h and entire p19.7 genes indicted the segregation of global isolates into six distinct clusters. In the HSP70h phylogeny all the global isolates having 544 specific sequences segregated into six lineages (Fig. 1 A). All the Indian isolates of this study clustered in group 2 except two isolates namely, Revella-4/12 and KS-B-7 which clustered separately along with TU 32 isolate from Tunisia (Fig. 1 A). One previously cloned Nashik isolate from India clustered in group 1. On the basis of complete p19.7 sequences, Indian isolates along with the global isolates of GLRaV-3 clustered into six different lineages (Fig. 1 B). Out of eleven Indian isolates, nine grouped in cluster group 2 while the two isolates, Nashik and Revella-4/14 grouped in cluster 1 and 5, respectively (Fig. 1 B). Thus, three GLRaV-3 isolates from India namely, Revella-4/12, Revella-4/14 and KS-B-7 showed different grouping behaviour based HSP70h and p19.7 phylogenies. On the basis of partial HSP70h nucleotide sequences, the Indian isolates showed maximum identities of 100 to 98.8 % with the isolates from India or elsewhere while the range for minimum identities were 73.4 to 72.5 %. The entire p19.7 nucleotide sequences identity matrix revealed the maximum identities of Indian isolates ranging from 100 to 94.8 % with the isolates from India or elsewhere while the range for minimum identities were 63.5 to 62.5 %.

Recombination analysis of p19.7 gene revealed the presence of Nashik isolate as recombinant with 6-18 isolate from USA and Manjri-A2-38/36 from India as the major and minor parental sequences, respectively. None of the Indian isolate

was found to be recombinant in HSP70h recombination analysis. The normalized values for the ratio of nonsynonymous substitutions per nonsynonymous site to synonymous substitutions per synonymous site (normalized *dN-dS*) values were 0.0865 for the HSP70h gene and 0.2303 for p19.7 gene. The results showed that both the genes are under purifying selection (normalized *dN-dS* < 1). It further indicated the contrasting selection pressure acting on the two genomic regions encoding proteins with distinct functions, with HSP70 being subjected to stronger functional constraints. The genetic variability of GLRaV-3 presented in the study provides a foundation for better understanding of grapevine leafroll disease across grape growing regions of India. This is the first description of genetic diversity and recombination in GLRaV-3 isolates from India. The genetic variability knowledge of GLRaV-3 will benefit in improving the sanitary status of grapevine planting materials in the country after elucidating the biological and epidemiological implications of genetic diversity, which in turn will provide the avenues for the development of robust strategies for mitigating the negative impacts of grapevine leafroll disease.

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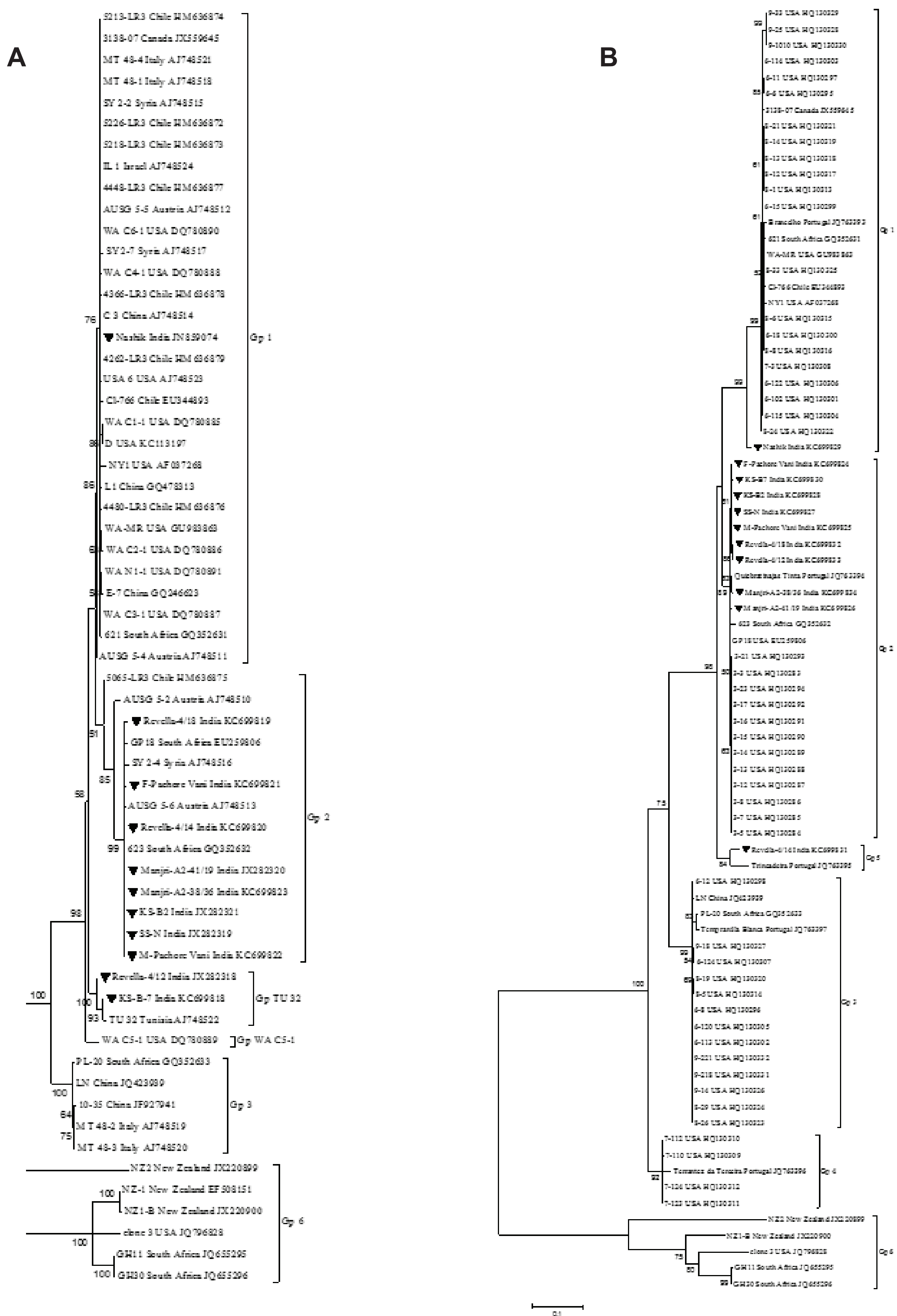

Figure 1: Phylogenetic analysis of global isolates of *Grapevine leafroll-associated virus 3* based on nucleotide sequence of the partial HSP70h (A) and p19.7 gene (B).The isolates under study have been marked by solid triangles.

PP 14 - Genetic variability of *Grapevine leafroll associated virus 2* isolates from Argentina

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INTRODUCTION

Grapevine leafroll associated virus 2 (GLRaV-2) is the only member of the *Closterovirus* genus associated to Grapevine leafroll disease. Up to six monophyletic groups were defined in function of the coat protein (CP) sequence (Bertazzon *et al.*, 2010; Jarugula *et al.*, 2010), showing a high intraspecific variability. This species can be transmitted to herbaceous host, allowing the identification of four biological isolates based on symptomatology displayed by *Nicotiana benthamiana, N.clevelandii* and *N.occidentalis* (Goszczynski *et al.*,1996; Abou Ghanem-Sabanadzovic *et al.*, 2000). In this work we performed the genetic characterization of the tandem CP-p19-p24 of GLRaV-2's isolates present in Mendoza province, Argentina. At least one isolate from each phylogenetic cluster identified was used for mechanical transmission trials. We evaluated the relationship between transmission ability and the genetic variability of the isolates under study.

MATERIALS AND METHODS

From a total of 127 grapevine plants analyzed by DAS-ELISA, fifteen GLRaV-2 infected accessions were selected for dsRNA extraction (Zhang *et al*., 1998). An aliquot of dsRNA was submitted to RT-PCR (LR2-F/p24-R: ATGGAGTTGATGTCCGAYRR/ AGGTAGATACACCCACGTTCG) to amplify a 1.7 kb fragment corresponding to the genomic tandem CP-p19-p24. The PCR products were analyzed by RFLP using the TaqI enzyme in order to identify different genetic variants of GLRaV-2 (Lanza Volpe *et al*., 2015). Three clones for each genetic variant identified by sample were sequenced to assess their phylogenetic pertinence by maximum likelihood (ML) using PAUP*. Boostrap values were determined from 1000 replicates using Garli software. From the phylogenetic reconstruction, eleven accessions representatives of each GLRaV-2 group detected in this study were selected for mechanical transmission trials, according to Lanza Volpe *et al.* (2015).

RESULTS

ML-based phylogenetic analysis of the tandem CP-p19-p24 showed that Argentinean GLRaV-2 isolates belonged to four out of the six previously reported lineages (Fig. 1). Most of the isolates (17/20 genetic variants detected) belonged to the lineages PN and 93/955. The groups H4 and RG were represented by at least one isolate. Whereas some grapevine samples were infected by a single isolate, others were infected by mixture of two genetic variants of GLRaV-2 (samples Murv, RC, Pic, MB28, MB3). None of the isolates grouped into the lineages BD or Pv20. At least one isolate from each phylogenetic cluster was successfully transmitted to *N. bentamiana*. The ML tree inferred with the sequences obtained from grapevine and *N.benthamiana* confirmed the clustering of the isolates in four lineages (Fig. 1). In the cases where the grapevine plants showed mixed infections with differents genetic variants of GLRaV-2, only one was recovered from *N.benthamiana* (samples Murv and RC). Unexpectedly, in the samples PR, PN and SC, the isolate detected in the herbaceous host belonged to a different genetic lineage than the one identified in grapevine. The biological behavior of the different isolates transmitted to *N.benthamiana* showed similarities with the previous reports. Contrary to what was reported by Alkowni *et al.* (2011) we successfully transmitted an isolate that represent the RG lineage (isolate RGp). The genetic region analyzed doesn't appear to be involved in the transmissibility, since at least one isolate from each lineage detected in our culture region was successfully transmitted to *N. benthamiana*.

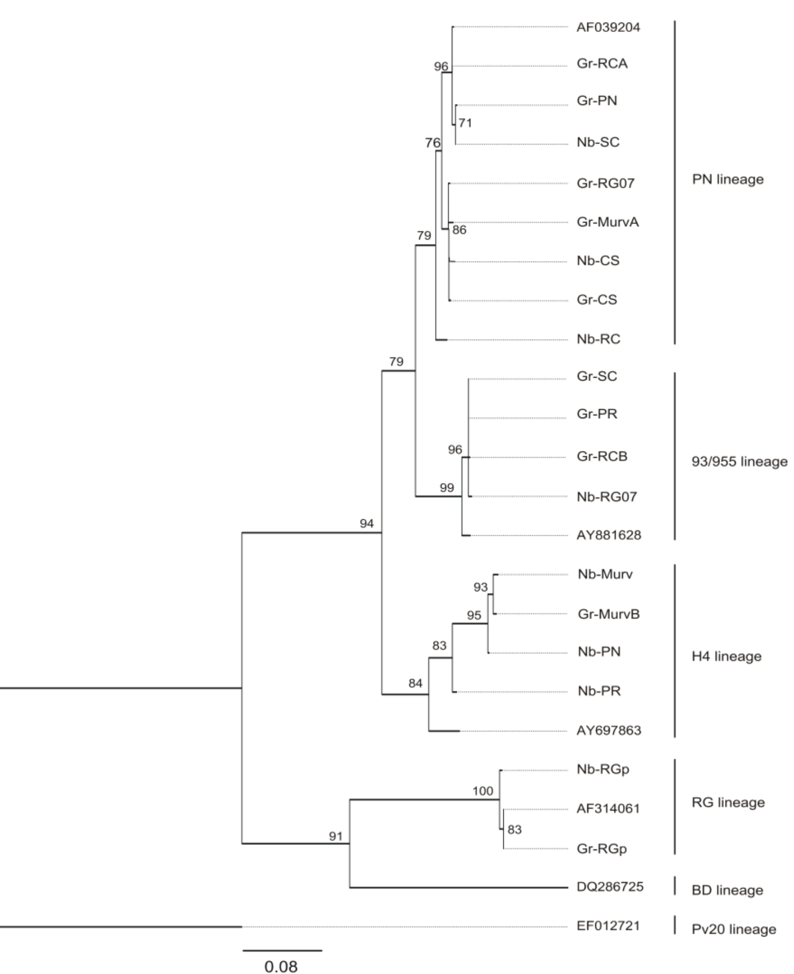

Figure 1. ML inferred tree performed with the sequences of the CP-p19-p24 tandem of the isolates under study. The prefix Gr- and Nb- followed by the name of the isolate, were used to identify the genetic variants recovered from grapevine and *N.benthamiana* infected tissues, respectively. The sequences obtained from grapevine samples presenting mixed infection with different genetic variants of GLRaV-2 are identified as A and B (Samples Pic, Murv, MB3, MB28, RC). The references sequences for each GLRaV-2 lineage are: AF039204 (GLRaV-2-PN), AY881628 (GLRaV-2-93/955), AF314061 (GLRaV-2-RG), AY697863 (GLRaV-2-H4), DQ286725 (GLRaV-2-BD) and EF012721 (GLRaV-2-Pv20).

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PP 15 - Biological Indexing of *Grapevine leafroll-associated viruses* (GLRaVs) in Aegean Vineyards, Turkey

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INTRODUCTION

Grapevine leafroll-associated viruses (GLRaVs) are one of the most important viral diseases of grapevine all over the world. Sap-transmitted viruses of concern belong in the genus *Nepovirus* and, to a lesser extent, to the genera *Vitivirus* and *Closterovirus* (Rowhani et. al. 2005). To date, 12 leafroll-associated viruses have been described as GLRaV-1, GLRaV-2, GLRaV-3, GLRaV-4, GLRaV-5, GLRaV-6, GLRaV-7, GLRaV-8, GLRaV-9, GLRaV-Pr, GLRaV-De and GLRaV-Car (Martelli et al., 2012). In spite of that only GLRaV-2 is mechanically transmissible to an herbaceous host (*Nicotiana benthamiana*) (Ling et al. 2007). This study was carried out to investigate the biological indexing of the leafroll-associated viruses into woody indicators and herbaceous hosts in Aegean vineyards.

MATERIALS AND METHODS

Virus Source

Twenty five isolates which were infected at least one GLRaVs were used for biological indexing on woody indicators while 13 of them were used on herbaceous hosts.

Woody Indicators

V. vinifera cv. Cabernet Franc, *V. vinifera* cv. Cabernet Sauvignon and *V. vinifera* cv. Pinot Noir were used for woody indicators of *Grapevine leafroll-associated viruses* (Martelli, 1993; Pearson and Goheen, 1988; Krake et al., 1999).

Herbaceous Hosts

Nicotiana occidentalis Weel., *Nicotiana benthamiana* Domin., *Nicotiana clevelandii*, *Chenopodium quinoa* and *Chenopodium amaranticolor* were used for herbaceous hosts of GLRaVs (Monette et al., 1990; Namba et al., 1991; Abou Ghanem-Sabanadzovic et al., 2000).

Biological Indexing on Woody Indicators

Dormant canes were removed from the infected grapevines. Then, cuttings with one bud was grafted with omega grafting technique onto each of six indicator plants. All indexing materials were incubated 25°C and 70%RH conditions in automatized climate room during 90 days for symptom development.

Mechanical Transmission into Herbaceous Hosts

Leaves and shoot tips were collected from infected grapevine plants freshly and removed petioles and main veins for mechanical transmission into herbaceous hosts. Mechanical transmission procedure were performed according to the referred publication (Rowhani et al. 2005). All sap-inoculated herbaceous host were incubated 25°C and 70%RH conditions in automatized climate room during 30 days for symptom development.

RESULTS AND DISCUSSION

Biological Indexing Results

All of 25 isolates which were infected with at least one *Grapevine leafroll-associated virus* were developed different severity symptoms on the indicator plant leaves (Figure 1).

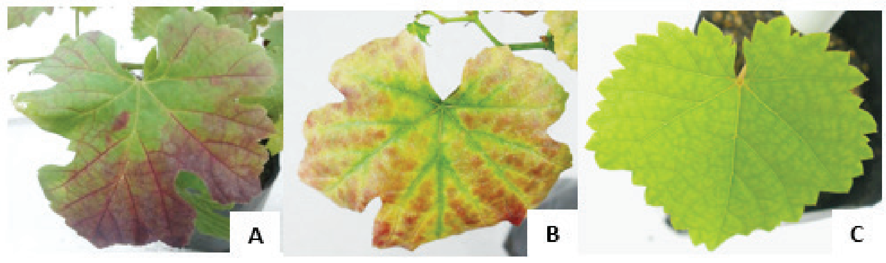

Figure 1. Symptoms development on different indicator plants were induced by isolate B21*.

A- Severe dark blotch coloration on leaf vein and blade on Cabernet Sauvignon. **B-** Severe clear blotch coloration on leaf blade on Pinot Noir **C-** No symptoms were observed on Cabernet Franc.
*Isolate B21 were infected with GLRaV-2, GLRaV-3, GLRaV-8 and GLRaV-De.

At the end of biological indexing assays, Cabernet Sauvignon were found to be most symptom indicative woody indicators followed by Pinot Noir and Cabernet Franc respectively.

Mechanical Transmission Results

Only one isolates were developed symptoms on herbaceous hosts out of 13 isolates. Isolate U5 which was infected with GLRaV-8 and GLRaV-De were developed chlorotic ringspot symptoms on *Nicotiana benthamiana* leaves (Figure 2).

Figure 2. Chlorotic ringspot symptoms on *Nicotiana benthamiana* leaves were induced by isolate U5 on the left and negative control as healthy leaf on the right side.

After the symptom development on *Nicotiana benthamiana*, RT-PCR tests were performed with specific primer pairs belong to GLRaV-8 and GLRaV-De for to detect which leafroll agent were caused this symptoms. However some studies about sequence of GLRaV-8 were showed that it's likely of the non-viral origin, also it's a part of grapevine genome according to the hypothesis of reference publications (Bertsch, et al., 2009 and Martelli et al., 2012); at the end of the RT-PCR assays, symptom indicated *Nicotiana benthamiana* leaves were found positive for only GLRaV-8 interestingly. Besides that, contrary previous research about mechanical transmission of leafroll-associated virus were observed no symptoms regarding to GLRaV-2 isolates on none of herbaceous hosts.

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PP 16 - Partial molecular characterization of *Grapevine leafroll-associated virus 4* (GLRaV-4) in Aegean vineyards, Turkey

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INTRODUCTION

Up to now, twelve *Grapevine leafroll-associated viruses* (GLRaVs) have been described (Martelli et al., 2012) and 11 of them (GLRaV-1, -2, -3, -4, -5, -6, -7, -8, -9, -Pr and –De) were reported in Turkey (Akbaş et. al., 2007; Buzkan et. al., 2010; Kaya et. al., 2012; Önder and Gümüş, 2014). GLRaV-4 is one of GLRaVs that has been firstly reported from Aegean viticulture areas in Turkey (Kaya et. al., 2012). In this study, three GLRaV-4 isolates which were C31, I1 and R73 deposited in Genbank with KP144369, KP144370 and KP144371 accession numbers respectively. GLRaV-4 isolates which were obtained from Aegean vineyards were characterized with their partial hHSP70 gene and constructed phylogenetic tree and similarity of nucleotide sequence with selected NCBI Genbank reference records.

MATERIALS AND METHODS

Isolate Source

Isolate C31, I1 and R73 were sampled from *V. vinifera* cv. Sultani Çekirdeksiz and collected in Salihli-Manisa, Kemalpaşa-İzmir and Çal-Denizli location (town-province) respectively. Total RNA was extracted with using “Zymo ZR Plant RNA MiniPrepTM” (Zymo Research Corp., USA) with little modifications on the manufacturer’s protocol. Later on, cDNA synthesis was derived via reverse transcription according to the “First Strand cDNA Synthesis Kit Protocol” (Fermantas, USA). cDNA amplification was carried out with FastStart Essential DNA Green Master (2x) (Roche®, Germany) and 20 pmoles of reverse (5’ CATACAAGCGAGTGCAATTACA-3’) and forward (5’ ACATTCTCCACCTTGTGCTTTT 3’) primers (Osman et al., 2007). Purified amplicons obtained from at the end of cDNA amplifications and purifications were sequenced bidirectionally with forward and reverse primers for GLRaV-4.

Sequence Data Analyses

The consensus sequences were generated from overlapping amplicons via Chromas Pro 1.7.6 analysis software. At the end of consensus sequences 319 nucleotides length were acquired for further phases. Later on, multiple sequence alignments of partial hHSP70 gene of GLRaV-4 isolates were performed using Bioedit 7.2.5. analysis software with ClustalW application as referred publication (Thompson et al., 1994). For multiple alignments, 14 NCBI Genbank records (JN226663, JN226662, GQ849394, FJ467503, AF039553, GU735409, KC202814, GQ246624, EU746619, KC202815, EU746618, KC113198, AM162280 and DQ325516) regarding with hHSP70 gene of GLRaV-4 were used for reference sequences. Finally, the nucleotide and protein sequence identities and phylogenetic analyses of all selected isolates were performed via MEGA 6.06 analysis software (Tamura et al., 2011). The phylogenetic relationships were determined with the neighbor-joining algorithm with Kimura 2 parameter model.

RESULTS AND DISCUSSION

Nucleotide and Amino Acid Identity

Multiple sequences alignments of C31 (KP144369), I1 (KP144370) and R73 (KP144371) were shown that high degrees of identity at the nucleotide level with the partial hHSP70 genes of China isolate (JN226663) of 97.87%, 97.16% and 97.87% respectively. While minimum identity degrees was 88.68% of Turkey isolate (DQ325516) with I1 (KP144370), followed by 89.44% of Israel isolate (AM162280) with C31 (KP144369) and R73 (KP144371) with same degrees. These isolates amino acid identity showed that C31 (KP144369), I1 (KP144370) and R73 (KP144371) were completely similar (identity degrees were 100%) with Chinia (JN226663, JN226662, GQ849394) and Spain (GU735409) isolates. Minimum amino acid identity degrees was 89.36% of United States (KC113198) and Israel (AM162280) isolates with C31 (KP144369), I1 (KP144370) and R73 (KP144371).

Phylogenetics Analyses

GLRaV-4 isolates were separated in two different subgroups. While, own sequenced isolates (I1, C31 and R73) with other referenced isolates were classified in Subgroup 1, Israel, USA and Turkey isolates (no informations about origin) were classified in Subgroup 2 (Figure 1). I1, C31 and R73 were classified more closely together.

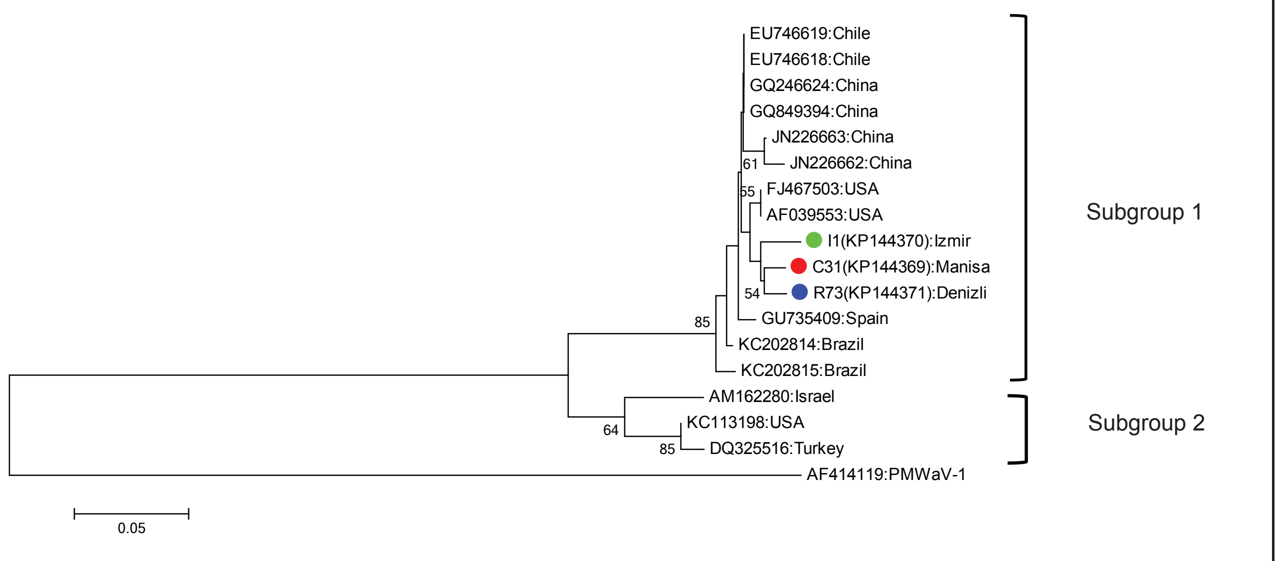

Figure 1. Phylogenetic tree constructed using partial hHSP70 gene nucleotide sequences of selected GLRaV-4 isolates. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. Only values with p>0,5 are shown. PMWaV-1 were used as an out-group.

Based on our results, C31 (KP144369), I1 (KP144370) and R73 (KP144371) were shown high genetic identity with the selected GLRaV-4 records in NCBI Genbank.

ACKNOWLEDGMENTS

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PP 17 - A novel ampelovirus from grapevine, and RT-PCR detection using universal primers for closteroviruses.

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INTRODUCTION

There are five recognized grapevine leafroll-associated viruses (GLRaVs) in the family *Closteroviridae*. GLRaV-1, -3, and -4 are ampeloviruses, GLRaV-2 is a closterovirus, and GLRaV-7 is a velarivirus (Maliogka et al., 2015). All of them can be detected by the nested RT-PCR using universal primers targeting heat shock protein 70 homolog (HSP70h) gene of the *Closteroviridae* (Dovas and Katis, 2003). Here, we describe a new putative ampelovirus which was detected using the nested RT-PCR. Also, we demonstrate RT-PCR detection of closteroviruses using mixture of universal primers originally designed.

MATERIALS AND METHODS

A nested RT-PCR using the universal primers targeting HSP70h of the *Closteroviridae* (Dovas and Katis, 2003) was performed using the method described by Nakaune et al. (2008) in one tree of *Vitis vinifera* (sample Ak177) showing typical leafroll symptoms (Fig. 1) in a NIFTS orchard. Nucleotide sequencing of the amplified fragments and subsequent 5′- and 3′-rapid amplification of cDNA ends (RACE) to determine the complete genome was performed following the methods of Ito et al. (2013).

RT-PCR to detect one of GLRaVs was carried out using a One-step RT-PCR kit (Qiagen). Specific primers to detect GLRaV-1 and GLRaV-3 were those listed by Nakaune and Nakano (2006). DAS-ELISA to detect GLRaV-1 was performed with the ELISA reagents for the detection of GLRaV-1 (Bioreba AG).

Several universal primers targeting HSP70h of the *Closteroviridae* were designed. They were mixed and used in a Superscript III One-step RT-PCR system with Platinum Taq (Invitrogen) to detect multiple members of the family.

RESULTS ANS DISCUSSION

The complete genome of the virus tentatively termed GLRaV-Ak consisted of 17,608 nt and encoded eight potential open reading frames, showing the characteristic organization of closteroviruses. RT-PCR showed that GLRaV-Ak was transmitted by grafting and GLRaV-Ak was detected from some trees in NIFTS orchards. However, because all of the trees were infected together with GLRaV-3, symptoms induced by GLRaV-Ak itself remain uncertain. The phylogenetic trees showed that GLRaV-Ak had the closest but distant relationship to GLRaV-1. DAS-ELISA targeting GLRaV-1 did not detect positive reaction from the sample Ak177 infected with GLRaV-Ak, which showed no possible serological relationships between them. GLRaV-Ak could be a new ampelovirus species whose relationship to grapevine leafroll symptoms should be further investigated. The RT-PCR using mixture of the universal primers detected several members of the *Closteroviridae* successfully (Fig. 2).

Figure. 1. Typical leafroll symptoms on the source vine

Figure 2. Agarose gel electrophoresis analysis of the fragments amplified in the one-step RT-PCR using mixed universal primers targeting HSP70h of the *Closteroviridae*. M: Marker; 1: 1: GLRaV-1/GLRaV-3; 2: GLRaV-1; 3: GLRaV-2; 4: GLRaV-3; 5: GLRaV-4/GLRaV-7; 6: GLRaV-3/GLRaV-7/GLRaV-Ak; 7: GLRaV-3/GLRaV-Ak; 8: Healthy grapevine; an arrow head: the specific band

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PP 18 - Investigation of newly-emerging grapevine viruses in The Central Anatolia region of Turkey

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INTRODUCTION

The grapevines (*Vitis* spp.) have been cultivated in Europe and Asia for thousands of years and have a highly valuable agricultural and economic importance. As most of the vegetatively propagated crops, grapevines are exposed to the attacks of a variety of viral agents which play a major role, causing heavy economic losses (Martelli 2014). The improvements on the "high-through output" or "next generation" sequencing technologies allowed the discovery of several unknown viruses. The identification of the putative Marafivirus *Grapevine Syrah virus 1* (GSyV-1) is the first example of the application of this novel technology in grapevine virology (Al Rwahnih et al. 2009). *Grapevine vein clearing virus* (GVCV), is the first identified DNA virus in *Vitis* and it is associated with a disease called 'Grapevine vein clearing and vine decline syndrome' (Zhang et al. 2011). The second virus, *Grapevine Pinot gris virus* (GPGV), was originally identified in a cv. Pinot gris plant showing a syndrome characterized by leaf mottling and stunting (Giampetruzzi et al. 2011). Also, a new virus *Grapevine red blotch-associated virus* (GRBaV), were identified with NGS (Al Rwahnih et al. 2013). Aim of this study was to investigate these newly-emerging viruses in the Central Anatolia region of Turkey.

MATERIALS AND METHODS

In the summer of 2014, 200 grapevine samples which were showing virus-like symptoms were collected from Central Anatolian region of Turkey. The RNAs were extracted using a commercial kit (Qiagen, RNeasy Plant Mini Kit) and cDNA was synthesized using random primers with the Super Script Choice System (Invitrogen, USA). PCR analysis was performed with virus specific detection primers of GLRaV1, GPGV, GRBaV and GSyV (Alabi et al. 2011, Rwahnih et al. 2013, Glasa et al. 2014, Maliogka et al. 2015).

RESULTS AND DISCUSSION

Leafroll 1, which is the most common virus diseases of the grapevines were used for preliminary analysis. Based on the survey analysis from Central Anatolian samples, none of tested viruses were detected, except GLRaV1. These results show that the tested grapevines from Central Anatolian region of Turkey are not affected by these newly-emerging viruses, yet. Further studies are under investigation.

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PP 19 - Recent spread of grapevine leafroll viruses in German vineyards: Spatial distribution of infected plants and identification of potential vectors

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INTRODUCTION

Grapevine leafroll disease (GLR) is one of the most widespread viral diseases in grapevine (*Vitis vinifera*) worldwide. It is caused by nine different viruses of the genera Closterovirus and Ampelovirus which are named grapevine leafroll associated virus (GLRaV) -1 to 9. In Germany grapevine leafroll disease is most commonly caused by GLRaV-1.

In the 1930s estimated 80% of German vines were infected, in some varieties close to 100% (Scheu, 1935). GLR was almost eradicated during the last 70 years by clonal selection and consistent elimination of infected vines. These measurements resulted in drastically lowered infection rates and accordingly to higher yields and fruit quality. Since 2013 this success story is at risk, as spreading of leafroll onto formerly healthy plants was observed in several vineyards, indicating the presence of vectoring insects. Until then it was assumed that only infected grafting material was responsible for the spread of GLRaV-1 in Germany.

The aim of this study is the analysis of the spatial distribution of newly infected vines and of the insect fauna in the areas of new GLRaV-1 infections. Frequently occurring insects with a piercing-sucking mode of feeding were assayed for GLRaV-1 as a first step towards the identification of insect vectors of GLRaV-1 in Germany.

MATERIALS AND METHODS

Studies were conducted in several mother blocks of the Institute for Grapevine breeding, Geisenheim University, in various parts of Germany. Leaves of plants in areas surrounding vines well known to be infected, were tested for the presence of GLRaV-1 particles. Tests were carried out by ELISA and a PCR-based method (Frotscher et al., 2015).

In the area of GLRaV-1 spread, phloem sucking insects were sampled, as they represent potential vectors for GLRaV-1. The insects were tested for the presence of GLRaV-1 particles. For this purpose, a PCR-based method for the detection of GLRaV-1 in grapevine (Frotscher et al., 2015) was adapted to detect the virus in insects.

RESULTS AND DISCUSSION

The distribution of newly infected vines is scattered and located on the eastern side of GLRaV-1 infected plants. This pattern indicates an aerial vector, following the prevailing wind from west.

The PCR based assay developed in this study proofed to be a valuable tool for the detection of GLRaV-1 in insects. It requires very low sample volumes and an internal positive control (18sRNA) helps to avoid false negative results. The assay was used to identify additional candidate vectors of GLRaV-1 which will subsequently be assayed for their ability to transmit GLRaV-1 from infected to healthy grapevines in transmission experiments.

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PP 20 - First report of *Grapevine rupestris stem pitting-associated virus* (GRSPaV) in autochthonous grape varieties in Turkey

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INTRODUCTION

Grapevine rupestris stem pitting-associated virus (GRSPaV), which is a member of the genus *Foveavirus* (Martelli and Jelkmann, 1998) in the newly established family *Betaflexiviridae* (Martelli et al., 2007). Viruses in this genus have filamentous particles *ca*. 800 nm long, with *Apple stem pitting virus* (ASPV) as the type species. GRSPaV is a positive sense ssRNA virus, 8.7 kb in size and polyadenylated at the 3' terminus (Martelli and Jelkman, 1998). The virus is usually detected in *Vitis vinifera* L. cultivars in a latent state; this means that cultivating symptomless but GRSPaV-infected vines carries a risk of the development of RSP via grafting. Information on the presence and variability of GRSPaV in turkish vineyards is not available yet although the virus is listed as certification pathogen (http://www.tarim.gov.tr/Belgeler/Mevzuat/Talimatlar/BUGEM). Therefore, we have initiated a comprehensive study to document the prevalence and genetic variability of GRSPaV in autochthonous cultivars in two important grape regions, Eastern Mediterranean (EM) and Southeast Anatolia (SEA).

MATERIALS AND METHODS

A total of nineteen autochthonous varieties was investigated in 34 vineyards (Table 1). No clear symptoms attributable to rugose wood disease were observed on the trunks and this not common situation could be mainly explained with prevalence of self rooted vineyards/plants visited. One-year old cuttings were collected for GRSPaV detection. Phloem scrapings was used for total nucleic acid isolation with silica-capture method (Foissac *et al.,* 2005) and two-step reverse transcription polymerase chain reaction (RT-PCR) was performed using primers RSP 13/RSP 14 (Meng *et al*., 1999). PCR amplicons were sequenced directly with primer RSP 14 by Macrogen (Netherlands). Alignments of the obtained sequences with additional sequences retrieved from GenBank after using the Blastn program (Altschul *et al*., 1997) and MEGA v.05 software (Tamura *et al*., 2013).

Table 1. Surveyed areas and turkish autochthonous grape varieties

Region	Location		No of vineyards	No of positive/ tested samples	Autochthonous varieties
Eastern Mediterranean	**Mersin**	Ulaş (Tarsus)	4	7/16	Yalova incisi, Tarsus beyazı, Ergin çekirdeksiz, Trakya ilkeren
		Kaleburcu (Tarsus)	6		Yalova incisi
	Adana	Dokuztekne (Ceyhan)	3	9/13	Yalova incisi, Trakya ilkeren, Dokuztekne karası
	Hatay	Söğütlü (Hassa)	3	2/8	Pafu, Hönüsü
Southeast Anatolia	**Gaziantep**	İslahiye	2	2/5	Hatun Parmagı, Horoz Karası
	Şanlıurfa	Gülpınar (Hilvan)	1	4/6	Çiloreş, Azezi, Küllahi
		Diphisar (Hilvan)	1		Çilorut, Sergi karası (syn: Cibine)
	Adıyaman	Köseceli (Besni)	2	0/7	Besni, Tahannebi, Peygamber üzümü
		Ovacık (Besni)	2		Besni
	Kilis	Akçabağlar	2	13/51	Rumi, Sultani Çekirdeksiz
		Kurukastel	5		Horoz karası, Rumi
		Ovacık	3		Horoz karası, Rumi

RESULTS

Approx. 35% of the tested plant samples resulted positive for GRSPaV (Table 1). The high number of GRSPaV-infected samples in SEA region was obtained from Kilis and Adana was another location from where GRSPaV was mostly detected in EM. Comparative analysis of 17 Turkish (TK) and other GRSPaV isolates showed close relationship. The identity of nucleotides of the sequenced amplicon among the isolates ranged between 87% and 96%. Two clustering patterns could clearly be seen in the phylogenetic analysis (Fig. 1) made by Neighbour-Joining method. Group I contained the reference strains SG1 (*Vitis rupestris* cv. Saint George) (Meng *et al*., 2005) and MG (*V. vinifera* cv. Moscato Giallo) (Morelli *et al*., 2011) which were in close relationship with TK11 derived from cv. Rumi in SEA. Group II had three branches with the reference strains RSPV-1 (Meng *et al*., 1998), GRSPV and GRSPaV-BS (Bertille Seyve 5568, French-american hybrid grapevine). The first branch contained a strain from Canada (JX513892) in close relationship with RSPV-1 and GRSPV. The composition of viral variation of the isolate TK105 derived from cv. Rumi at SEA was slightly different from all others. The interspersed allocation of the GRSPaV type-strains along the phylotree does confirm that the quasi-species worldwide distribution of the virus genome is mirrored also in the Turkish accession, notwithstanding their long lasting self rooted condition and local management.

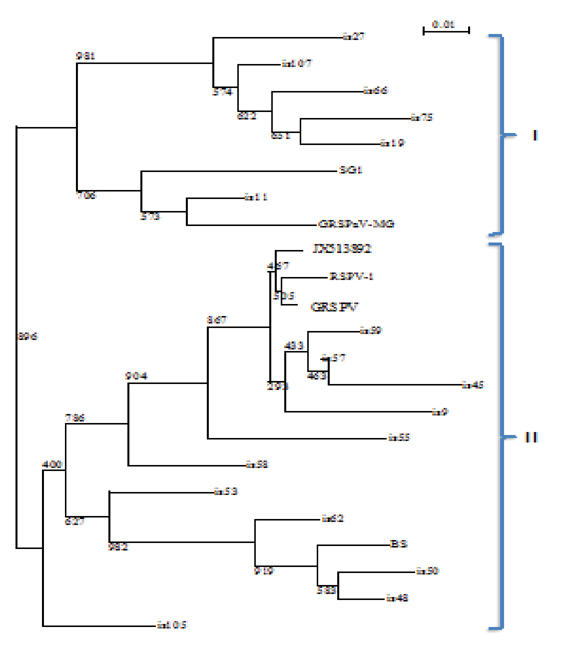

Figure 1. Genetic variability and comparison of Turkish GRSPaV isolates with type strains of the virus; RSPV-1 (AF057136), GRSPV (AF026278) and GRSPaV-BS (AY881627). The robustness of tree topology was evaluated with 1,000 bootstrap resamplings.

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PP 21 - First report of *Grapevine rupestris stem pitting associated virus* (GRSPaV) in Turkey

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INTRODUCTION

Grapevine rupestris stem pitting-associated virus (GRSPaV), member of the genus *Foveavirus*, family *Betaflexiviridae* (Meng et al., 1998; Zhang et al., 1998; King et al., 2011) is the most prevalent virus of grapevines and has a worldwide distribution. It is linked with the complex Rugose Wood disease which affects both graft take and longevity of vines in a productive vineyard. This virus has only been detected in *Vitis*, and is present in over 90% of grapevine samples tested in different grapevine growing areas of the world. There has been no any report of RSPaV in Turkey up to now. Given the paucity of the presence and incidence of the virus in Turkey, an investigation was initiated, the preliminary results of which are reported herein.

MATERIALS AND METHODS

Field inspections and collection of samples were conducted in January 2015. Dormant canes were mainly collected from some local and foreign cultivars like Antep karası, Pafu, Chardonnay, Pinot Noir, Zinfandel and as well as unknown cultivars grown in different provinces of Turkey. All collected plant samples were tested for GRSPaV by RT-PCR. Total RNAs were extracted from 100 mg of cambium scrapings according to the Manufacturer (Qiagen RNeasy Mini Kit, Qiagen, Valencia, CA). GRSPaV spesific primer pairs RSP48 (AGCTGGGATTATAAGGGAGGT) and RSP49 (CCAGCCGTTCCACCACTAAT) (Zhang et al., 1998) targeting a 329 bp segment on the virus coat protein gene was used in a single tube RT-PCR. PCR fragments were visualized using ethidium bromide in 1.2% agarose gels in TAE buffer.

RESULTS AND DISCUSSION

The plants showing distortion, discoloration, downward rolling and mottling on the leaves were labelled during the growing season and they were sampled together with the symptomless plants in the fallowing dormant period for the molecular analysis. The RT-PCR results indicated the presence of GRSPaV in most of the symptomatic and some of the symptomless grapevine samples and expected size of amplicons (329 bp) were obtained (Fig.1). All tested local and foreign grapevine cultivars were found to be infected by GRSPaV. Among the tested 66 grapevine samples, 46.97% was found to be infected by this virus. According to RT-PCR analysis, this is the first report of GRSPaV in different grapevine cultivars in Turkey. The results documenting the occurrence of different viruses in grapevine cultivars improved our understanding of the sanitary status of vineyards in Turkey. Grapevine planting material produced in Turkey lacks adequate sanitary control. Clonal selection and implementation of sanitation program according to the requirements of EU certification standards is therefore urgently needed. Also, genetic diversity studies of these isolates are still in progress.

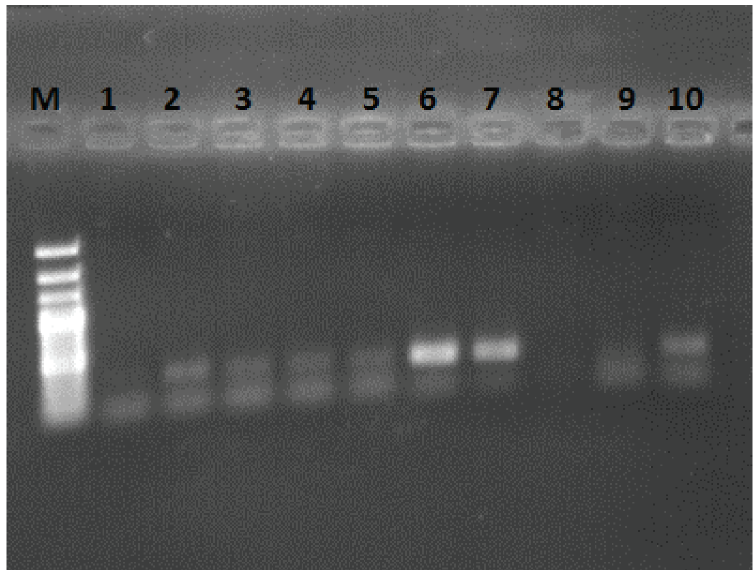

Figure 1. Agarose gel electrophoresis analysis of reverse-transcription polymerase chain reaction tests for specific detection of Grapevine rupestris stem pitting-associated virus (lane 1-7), healthy grapevine (Lane 8), water control (Lane 9) and positive control (lane 10). Lane M, Marker 100 bp Plus DNA Ladder (Fermentas, #SM1153).

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PP 22 - *Grapevine rupestris stem pitting-associated virus* improves tolerance to water stress and miRNAs are involved in these virus-plant-drought interactions.

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INTRODUCTION

Grapevine rupestris stem pitting-associated virus (GRSPaV) is one of the most prevalent viruses that infects grapevines and belongs to the genus Foveavirus, in the Betaflexiviridae family (Martelli *et al*., 2007). We have shown previously that plants of *V. vinifera* cv Bosco infected by the GRSPaV-1 sequence variant exerted a very moderate decrease in physiological efficiency and yield. Importantly, this response overlapped with molecular responses to water and salinity stress (Gambino *et al*., 2012). These considerations prompted us to investigate possible interactions between GRSPaV and abiotic stress. The molecular basis of these interactions is still poorly understood; however, it is reasonable to hypothesise an involvement of RNA silencing, which is a natural defence against invading viruses in plants. Both miRNAs and siRNAs are involved in several developmental processes in defence responses to biotic and abiotic stresses and in genome stability maintenance, as reviewed in many studies (Sunkar *et al*., 2007). We analysed the ecophysiological and molecular interactions between GRSPaV infection and drought in grapevine.

MATERIALS AND METHODS

The study was performed on the Italian white grape cultivar Bosco using self-rooted plants grown in pots in a greenhouse. Woody material was collected in the field from previously identified GRSPaV-infected ('infected plants' hereafter) and GRSPaV-free plants (Gambino *et al*., 2012). All 'Bosco' plants were derived from vegetative propagation from a single mother plant originally infected by GRSPaV and were further subjected to sanitation. Six GRSPaV-free and six infected plants were monitored during water deprivation by daily measuring of leaf gas-exchange parameters and stem water potential (Ψstem). Following the progressive decrease of the ecophysiological performances, leaves for molecular analyses (siRNA libraries and qRT-PCR) were collected on the base of three selected levels of stress: well watered (WW), water stress (WS: stomatal conductance, gs ≈ 60 mmol H_2O $m^{-2\,s-1}$; Ψstem ≈ -1MPa) or severe water stress (SWS: gs ≈ 25 mmol H_2O $m^{-2\,s-1}$; Ψstem ≈ -1.5MPa). In addition, we performed a second experiment in which the growth rate of six GRSPaV-free and six infected 'Bosco' plants grown in pots in a greenhouse and subjected to a moderate water stress treatment (-0.3 > Ψsoil > -0.1MPa; 20–32% soil water content) for 30 days were measured. Low-molecular-weight RNA was extracted from a pool of leaves from infected and GRSPaV-free grapevines in WW and SWS conditions. Libraries of sRNAs were then produced using a TrueSeq Small RNA Sample Kit (Illumina, San Diego, CA, USA) and sequenced using the HISeq 2500 Illumina platform. The identification of grapevine transcripts targeted by miRNAs was performed using CleaveLand software and by 5′-rapid amplification of cDNA ends (5'-RACE). The sequencing data were validated by qRT-PCR analysis following the protocol of Shi and Chiang (2005) with some modifications.

RESULTS AND DISCUSSION

During the progressive drying of soil, the stomatal conductance (g_s) and net photosynthesis (P_n) of both infected and GRSPaV-free sets of plants decreased in response to water stress; however, this response was delayed in infected plants (Fig. 1). The stomata of GRSPaV-free plants were less regulated throughout the whole experiment, and infected grapevines developed leaves with a significantly higher stomatal density than GRSPaV-free plants (Fig. 1). Prolonged drought conditions are known to reduce or block growth. However, in both irrigated and water stress conditions, infected plants showed a greater leaf area, height, and stem growth diameter compared to GRSPaV-free plants (Fig. 1). In the small endogenous RNAs libraries produced from leaves of infected and GRSPaV-free plants under WW and SWS, we identified members of almost all known miRNAs and 25 novel miRNA candidates. The analysis of the data revealed that the accumulation of some known miRNAs was clearly associated with GRSPaV infection. In addition, when GRSPaV-infected plants were subjected to water stress, the ratio of miRNA accumulation between infected and GRSPaV-free grapevines changed significantly. Interestingly, this study clearly shows that the sanitary status of the plants may play a determinant role in miRNA regulation

during abiotic stress. In infected grapevines, we observed the regulation of several miRNAs and their targets involved in leaf development (miR156, miR164, miR319, miR394, miR396). For example, in GRSPaV-free grapevines in response to high levels of miR396 and to decreases in the transcript level of *VvGRF5* in leaves (Fig. 2), we observed a reduction of stomata numbers, in line with activity shown for *Arabidopsis*. We have demonstrated that the novel miRC121 and miRC129 control of *VvLHC* and *VvPSI*, respectively, and the combinations *VvLHC*/miRC121 and *VvPSI*/miRC129 might be positively correlated with the higher levels of photosynthesis observed in infected plants (Fig. 2). The *NAC* genes play important roles in abiotic stress responses in several species and are generally up-regulated in grapevines following water stress, interact with abscisic acid (ABA) and represent a node between various signalling pathways. In our experiments, we observed that *VvNAC05* and *VvNAC11* (targets of miR164 and miRs409712_2, respectively) were induced by water stress. Furthermore, they were induced more rapidly than in GRSPaV-free grapevines (Fig. 2), and they showed the same expression pattern of *VvNCED* (a gene linked to the biosynthesis of ABA). The multiple regulation that controls the transcription of these *NAC* genes (post-transcriptionally regulated by miRNAs and transcriptionally regulated probably by ABA) induced a rapid response to drought in infected plants that might be positively linked to drought tolerance.

Our data support the idea of mutual adaptation between GRSPaV and grapevine, resulting in beneficial effects for the host under water stress conditions. Therefore, GRSPaV might represent the first example of a plant virus that is more appropriately defined by the categories of 'conditional mutualism' and 'beneficial virus' proposed by Roossinck (2011).

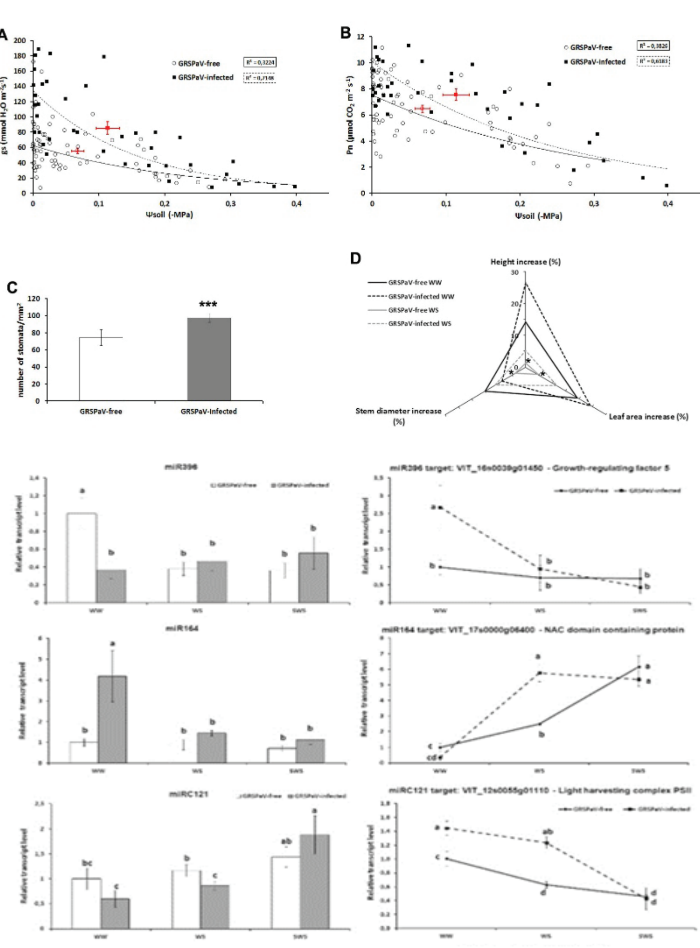

Figure 1. Relationship between (A) the stomatal conductance (g_s) and soil water potential (Ψsoil) and between (B) net photosynthesis (P_n) and Ψsoil in infected and GRSPaV-free plants. (C) Number of stomata in leaves, and (D) increased rates (%) in height, stem diameter and leaf area in GRSPaV-infected and free plants. *, ***= significant at 0.05 and 0.001, respectively.

Figure 2. Relative expression levels of miR396, miR164, and miRC121 and their respective targets in GRSPaV-free and infected 'Bosco' leaves as determined by qRT-PCR. Samples were collected under well-watered (WW), water stress (WS) and severe water stress (SWS) conditions. qRT-PCR signals were normalised to U6 and 5.8 rRNA for miRNA quantification, and to actin and ubiquitin transcripts for target quantification. Data are presented as mean ± standard error of two biological and three technical replicates (n = 6); different letters denote significant differences at p ≤ 0.05.

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PP 23 - Improved RT-PCR detection and prevalence of GVA, GVB and GRSPaV in Greek vineyards

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INTRODUCTION

Grapevine virus A (GVA), *Grapevine virus B* (GVB) and *Grapevine rupestris stem pitting associated virus* (GRSPaV), have a worldwide distribution and are closely associated with the Rugose wood (RW) syndrome (Martelli,1993, Martelli and Boudon-Padieu, 2006). A crucial step towards the effective control of these pathogens is their reliable detection through sensitive assays. So far the identification of these viruses with serological and molecular methods is problematic due to their high intraspecies variability. Therefore, the aim of this study was to develop new reliable RT-PCR assays for the detection of different variants of the three viruses which were then applied for the estimation of their frequency in grapevine varieties and rootstocks cultivated in Greece.

MATERIALS AND METHODS

Two step RT-PCR assays were developed, using degenerate primers that target the capsid protein (CP) gene of each virus. For the design of each primer pair all virus sequences available in the databases were used and the most conserved regions of the coat protein genes were selected. Several primers were evaluated before the final selection of those exhibiting the broadest detection range. The evaluation of the designed primers and the optimization of the developed methods were made using characterized virus isolates from different countries. More specifically, 10 isolates of GVA and GRSPaV and 11 of GVB were used. Total RNA was used as template in the reactions and it was extracted from cambial scrapings using the protocol of Maliogka et al. (2015) with the addition of 5% PVPP at the lysis buffer. RT was conducted using primers RSPaV_CP_487 and GVA_CP_R6864 for GRSPaV and GVA, respectively and GVB_CP_RT for GVB (Table 1) at a final concentration of 1,5 μM. Two μl of each cDNA were further used in PCR assays along with 1,5 μM of each of the respective primers (Table 1). Amplifications were done using an annealing temperature of 50 ^{o}C for GVA and GVB and 56 ^{o}C for GRSPaV. The developed RT-PCRs were applied for further study of the occurrence of GVA, GVB and GRSPaV in Greek vineyards. In total, we tested 156 samples of own-rooted indigenous varieties, 150 samples of grafted varieties and 25 samples of American rootstocks.

Table 1. Primers used for the detection of GVA, GVB and GRSPaV

Virus	Primer name	Targeted gene	Sequence (5'-3')	Amplicon
GRSPaV	RSPaV_CP_333	CP	CTC TGA YGT KGG HAG CTC YC	176bp
	RSPaV_CP_487		TTA GCK GGT GGW ATY CCC GTC TC	
GVA	GVA_CP_F6527	CP	AAY ACT CYC TTC GGG TAC ATC	340bp
	GVA_CP_R6864		GCR AAR TCR AAC ATA RCC TG	
GVB	GVB_CP_RT	CP	CCH GAG TTR AAR TCR A	343bp
	GVB_CP_F1		TTCAGRACCYTVTTYGGGTAC	
	GVB_CP_R1		CCH GAG TTR AAR TCR AAC ATN AC	

R:A/G, K:G/T, Y:T/C, H:A/C/T, V:A/C/G, N:A/T/G/C, W:A/T

RESULTS AND DISCUSSION

The developed RT-PCR assays exhibited a wide detection range since they were able to amplify all the different characterized isolates of GVA, GVB and GRSPaV tested in this study either alone or in mixed infections (Figure 1). Further sequencing of one amplicon from each RT-PCR confirmed the specificity of the reactions.

Testing of the samples derived from own-rooted varieties showed that GVA occurs in high frequency (37%), while GRSPaV and GVB were detected in smaller percentages (11% and 10%, respectively). In grafted varieties, GVA and GRSPaV occur in particularly high percentages (44% and 38%, respectively) while the presence of GVB remains low (11%). In the samples from the American rootstocks, the presence of GRSPaV was dominant (31%), followed by GVA (4%), while GVB was not detected in any of the samples.

Overall, it seems that grafting has increased the GVA and GRSPaV infection rates of the cultivated varieties while of particular interest is the presence of GRSPaV in own-rooted varieties given that no vector is known so far.

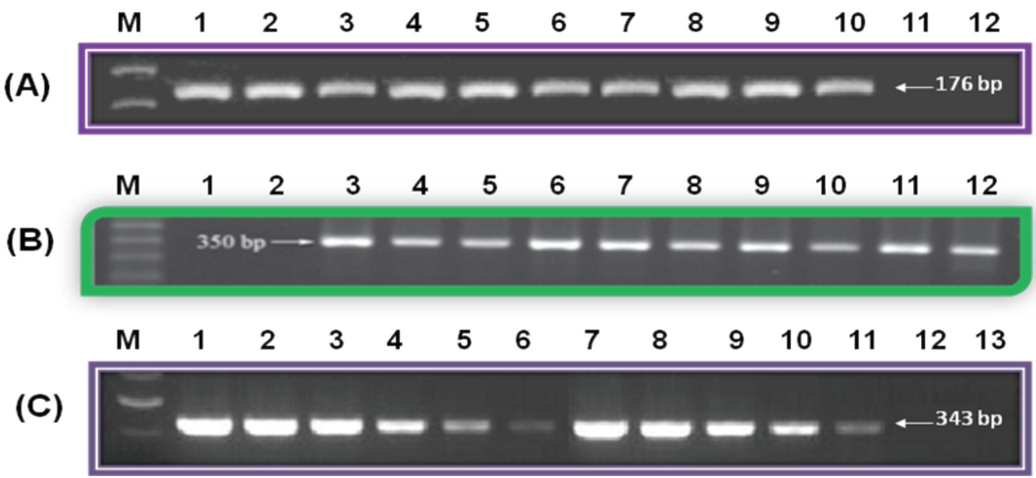

Figure 1. Agarose gel electrophoretic analysis of RT-PCR products obtained using RNA from isolates of GRSPaV (A), GVA (B) and GVB (C). (A) Lanes 1-6: isolates from Cyprus, 7-10: isolates from Greece, 11, 12: negative controls. (B) Lanes 1, 2: negative controls, 3-5: isolates from Cyprus, 6-8: isolates from Italy, 9: isolate from Spain, 10: isolate from Israel, 11, 12: isolates from Greece. (C) Lanes 1,2: isolates from Cyprus, 3-6: isolates from Italy, 7, 8: isolates from Spain, 9, 10: isolates from Israel, 11: isolate from Greece, 12, 13: negative controls. M: 100 bp ladder.

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PP 24 - Molecular detection of GVA and GVB variants in Portuguese grapevine varieties

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INTRODUCTION

Grapevine virus A (GVA) and *Grapevine virus B* (GVB) are both members of the genus *Vitivirus* in the family *Betaflexiviridae*. GVA is widespread in many grapevine-growing regions of the world (Du Preez et al., 2011), whereas GVB is much less studied. GVA has been implicated with Kober stem grooving disorder of the rugose wood disease complex (Minafra, 2000) and with Shiraz Disease (SD) in South Africa and Australia (Goszczynski and Habili, 2012). In turn, the clear-cut association of GVB with mild and severe symptoms of corky bark (CB) in the LN33 hybrid (Courderec 1613 x Thompson Seedless) strongly suggests that GVB causes CB (Whattam, 2003 and references therein).

Both viruses' filamentous particles are composed of a positive sense, single-stranded RNA with a genome encoding five open reading frames (ORFs1-5). Functions have been assigned for proteins encoded by ORF1 (virus replication), ORF 2 (suggested to be involved in transmission by mealybugs), ORF3 (movement protein), ORF4 (capsid protein) and ORF5 (10 kDa protein with putative nucleic acid binding property and RNA silencing suppressor activity) in the case of GVA (Galiakparov et al., 2003; Haviv et al., 2012; Zhou et al., 2006). Previous studies have indicated genetic diversity among GVA isolates (Goszczynski, 2007; Murolo et al., 2008; Wang et al., 2011), leading to segregation of virus isolates into three molecular variants, designated as Group I, II, and III. Some of these molecular variants have also been shown to have distinct biological properties. For instance, grapevines showing symptoms of SD have been consistently found to harbour molecular variants belonging to Group II in South Africa (Goszczynski, 2007) and Australia (Goszczynski and Habili, 2012), whereas isolates belonging to Group III were generally not associated with SD (Goszczynski, 2007). Fewer studies are available for GVB, but molecular analysis suggests that this virus is also extremely variable (Shi et al., 2004).

In this scenario investigating the genetic structure of GVA and GVB populations infecting field-established grapevines is vital to provide a background to improve detection and functional characterization of both viruses' variants.

MATERIALS AND METHODS

Plant material and virus source

The isolates analyzed in this work were each obtained from plant material collected from a different field-grown *Vitis vinifera* grapevine, at the National Collection of Grapevine Varieties (CAN PRT051) at INIAV (Dois Portos, Portugal). This collection, grafted on certified rootstock material (SO4, clone 73), was established 30 years ago on nematode-free soil, and is regularly tested for several viruses, either by DAS-ELISA, RT-PCR or PCR after cDNA synthesis. It is also maintained free of mealybugs. Several clone plants (i.e. vegetatively propagated from the same mother plant) represent each variety accession at CAN. The isolates here analyzed were obtained from the respective clone plant number 3 and tested positive in DAS-ELISA with commercial antibodies for GVA or GVB.

RNA extraction

Total plant RNA was extracted from each isolate with an E.Z.N.A.TM Plant RNA Kit (Omega Bio-Tek, USA), with the alterations to the manufacturer's protocol reported previously (Esteves et al., 2012).

cDNA synthesis and PCR

Synthesis of cDNA using a iScript cDNA First Strand Synthesis Kit™ (Bio-Rad, USA) was done according to the manufacturer's protocol in a final volume of 20 µl, with 5 µl of total RNA.

PCR reactions were performed in a final volume of 50 µl with Pfu DNA polymerase (Fermentas, Thermo Fisher Scientific, Inc.). The primer pairs initially tested in this work were retrieved from the literature pertaining to the detection and sequencing of GVA and GVB variants. Upon obtaining sequences for variants present in Portuguese isolates virus-specific primer pairs were designed for molecular detection purposes.

Cloning and sequencing

The amplicons obtained for each isolate, with different primer pairs, were ligated with the CloneJET™ PCR Cloning Kit (Fermentas, Thermo Fisher Scientific, Inc.) and used to transform *E. coli* XL1Blue (Agilent Technologies Inc., USA) competent cells. The PCR products of at least 16 positive clones per isolate were next analyzed by single-strand conformation polymorphism (SSCP). Recombinant clones evidencing different SSCP patterns were purified using an E.Z.N.A. Plasmid Miniprep Kit II (Omega Bio-Tek, Inc.), prior to commercial sequencing (Stab Vida, Caparica, Portugal).

Sequence data analysis

The sequences obtained for each isolate under study were visualized and aligned with homologous sequences retrieved from GenBank using BioEdit Sequence Alignment Editor (Bioedit) (Hall, 1999) and ClustalW (Thompson *et al*., 1994). Phylogeny was inferred using the Maximum Likelihood (ML) method implemented in MEGA5 (Tamura *et al*., 2011). Bootstrap values were estimated with 1000 replicates. Detection of evidence of putative recombination events was performed using the RDP v.3 software (Martin et al., 2010) and associated programs package.

RESULTS AND DISCUSSION

From an initial set of 30 isolates retrieved from Portuguese cultivars (CAN PRT051, Dois Portos), GVA nucleotide sequences extending through ORF3 to the 3'-UTR and GVB sequences encompassing ORF4 to 3'-UTR, were obtained from 8 and 4 isolates respectively, testing an array of published primers.

In the case of GVA (data shown on poster) the phylogenetic analysis, including homologous sequences from complete genome accessions available at GenBank, revealed the existence of two main lineages: one including groups I and II considered previously and another comprising group III. The ORF5 sequences, encoding the protein p10, also grouped into two well-resolved phylogroups (bootstrap>75%). The sequences retrieved from the Portuguese isolates grouped with homologous sequences available at GenBank, without evidence of segregation by geographical origin. Five of the isolates revealed infection by variants from different phylogroups. Several recombinant sequences were detected within the Portuguese isolates.

In the case of GVB the phylogenetic analysis conducted with sequences obtained in this work and homologous sequences from GenBank showed the existence of three main phylogroups (I-III), with the Portuguese variants distributed between groups I and II (Fig1). No recombinant sequence was found within the Portuguese isolates.

The molecular characterization of GVA and GVB heterogeneity from this study allowed the design of virus-specific primer pairs capable of amplifying all DAS-ELISA positive GVA and GVB isolates, which can be used for routine detection in a duplex-PCR assay.

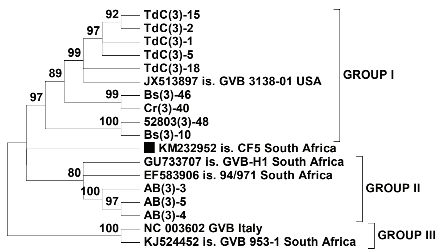

Figure 1. Phylogenetic analysis of GVB sequences (ORF4-3'UTR) retrieved from Portuguese isolates. The dendrogram was constructed by the maximum-likelihood (ML) method. Bootstrap values of 1000 replicates are shown at the nodes and branches reproduced in less than 75 % of bootstrap replicates are collapsed. Sequences retrieved from GenBank are indicated by accession number. Sequence KM232952 is. CF5 was found to carry evidence of recombination.

ACKNOWLEDGMENTS

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PP 25 - Characterizationof a novel reovirus species in Cabernet Grapevine in California

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INTRODUCTION

Grapevine, *Vitis vinifera,* supports an exceptionally diverse range of disease agents. Sixty eight different species of viruses have been identified in this crop (see Martelli, 2014, plus those listed here). Together with five known viroids, this gives grapevine the distinction of supporting a more diverse collection of subcellular pathogens than any other of the world's agricultural crops. Next Generation Sequencing (NGS) provides a powerful approach for the identification of viral pathogens in grapevine. The capacity of NGS to characterize the virome of an infected grapevine was first demonstrated by Al Rwahnih et al. (2009). In that case the technique identified the previously unknown Grapevine Syrah Virus 1, against a background of much higher titers of *Grapevine rupestris stem pitting-associated virus* (GRSPaV) and Grapevine rupestris vein-feathering virus. Further discoveries of undescribed grapevine viruses by NGS include that of Grapevine vein clearing virus (Zhang et al., 2011), Grapevine red blotch-associated virus (Al Rwahnih et al., 2013), Grapevine virus F (Al Rwahnih et al., 2012), Grapevine Pinot gris virus (Giampetruzzi et al., 2012) and Grapevine Roditis leaf discoloration-associated virus (Maliogka et al., 2015).

Here we describe the application of NGS technology to the discovery of a novel grapevine reovirus. The virus was discovered in a mixed infection, through the bioinformatics analysis of a dsRNA fraction isolated from a Cabernet Sauvignon vine in California (Al Rwahnih et al 2015).

MATERIALS AND METHODS

The source vine (*Vitis vinifera*) was a Cabernet Sauvignon grapevine (accession CS-Tok3). The disease symptoms in this host were those of severe grapevine leafroll disease. The reoviral genome was characterized from a total nucleic acid extract of bark scrapings that was enriched for double-stranded RNA (Al Rwahnih et al. 2009). A complementary DNA library was constructed from that extract and sequencing data was generated by SeqMatic LLC (Fremont, CA) using the Illumina HiSeq 2000 platform, after the removal of host genome sequences by mapping against the grapevine genome. Unmapped reads were analyzed using NCBI's BLASTx program (http://www.ncbi.nlm.nih.gov/ BLAST) to identify viral genomic sequences. That program was also used to compare the Grapevine reovirus genome against the genomes of other phytoreoviruses. A specific PCR assay was developed for the detection of this reovirus in grapevine material, from the sequence of viral genomic component 4. The specific PCR primers had the following sequences:

Ctg 468F (5`ACGTTGGATCAACTAGCCGAAG3`);

Ctg 468R (5`TATTCACGAGGCTCAGACGACT3`). PCR analysis (Al Rwahnih et al, 2009) using those primers was carried out on the total nucleic acid fraction extracted from bark scarping and petiole material as template. That PCR analysis was used to confirm the presence of the reovirus both in the source plants, and in Cabernet franc plants that had been bud grafted with the Cabernet Sauvignon source plant.

RESULTS AND DISCUSSION

The reovirus was initially found as a member of a mixed infection containing Grapevine leafroll associated viruses -2 and -3, Grapevine virus A, (GRSPaV), and Grapevine fleck virus. The disease symptoms in this Cabernet Sauvignon host were those of severe grapevine leafroll disease. Polyacrylamide gel analysis of the dsRNA fraction isolated from the host plant showed a prominent series of dsRNA bands in the 1 to 18 Kbp size range. Analysis of the sequences of the cDNA library generated from that dsRNA fraction revealed that about 18% (5,408,279) of the reads unmapped to the host were homologous to the reovirus sequence in the BLAST analysis. The genomic coverage of those reads was sufficient to contain sequence information of the ten reoviral genomic components. The genomic sequences have been deposited in the GenBank under the provisional name Grapevine Cabernet Sauvignon Reovirus (GCSV) and were assigned accession numbers KM236567, andKM378720 through KM378728. The sequence homologies of those components with the components of other phytoreoviruses (Table 1) ranged from 66 to 30% for Raspberry latent virus (RpLV), and from 57 to 22% for Rice ragged stunt virus (RRSV), indicating that the novel grapevine reovirus is a distinct viral species.

Table 1. Reovirus genomic segment inferred translation product sequence identities in the BLASTX comparison of GCSV against RpLV and RRSV, respectively. Sizes are for GCSV segment RNA sequences that have been submitted to GenBank.

RNA Segment	size (nt)	Accession no.	Blastx Identity (%)	
			RpLV	RRSV
S 1	3328	KM236567	55	57
S 2	3757	KM378720	62	28
S 3	3861	KM378721	67	38
S 4	3936	KM378722	66	26
S 5	2349	KM378723	52	25
S 6	1968	KM378724	35	-
S 7	1974	KM378725	30	-
S 8	1949	KM378726	63	26
S 9	1239	KM378727	54	22
S 10	1139	KM378728	49	24

The PCR primers designed from the viral sequence generated in the NGS analysis were used to confirm the presence of the virus in the Californian Cabernet Sauvignon source plant. A single 368 bp PCR product was produced in that analysis, sequenced in both directions and found to share 100% sequence identity with GCSV genomic segment 4, against which the primers were designed.

This specific PCR analysis was used to test for the transmissibility of GCSV from the original source plant to a Cabernet franc host that had been bud chip inoculated from the source plant. The test did demonstrate graft transmissibility of this reovirus. The PCR analysis has also recently been used to demonstrate the presence of GCSV in an infected grapevine in the state of Rio Grande do Sul, Brazil (Fajardo, T. personal communication).

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PP 26 - First Report of *Australian grapevine viroid* (AGVd) in the Aegean Region Vineyards of Turkey

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INTRODUCTION . . . .

Australian grapevine viroid (AGVd) falls within *Apscaviroid* genus (*Pospiviroidae*) which has 369 nt length (Rezaian, 1990; Randless, 2003). This viroid generates in combination with other grapevine viroids (like GYSVd-1, GYSVd-2, CEVd-g and HSVd-g) and it causes symptoms such as mottling, stunting, leaf deformation and vein clearing in its experimental hosts (Zaki-Aghl, 2013). AGVd has been reported in only six countries in the world: Australia (Rezaian, 1990), China (Jiang et al., 2009), Tunisia (Elleuch et al., 2002, 2003), the United States (Al Rwahnih et al., 2009), Iranian (Zaki-Aghl, 2013) and India (Adkar-Purushothama et al., 2014).

MATERIALS AND METHODS

Plant Samples and RT-PCR Analyses

Fifty seven plant samples were collected from different vineyards from Aegean region known as heavly infected by grapevine viroids. Collected grapevine leaf samples belonging to 20 different cultivars were analyzed by RT-PCR using specific primers to AGVd (Jiang et al., 2009) (forward 5'-ACC TGC AGG GAA GCT AGC TGG GTC-3' and reverse 5'-CCC TGC AGG TTT CGC CAG CAA GCG C-3').

Molecular Cloning and Sequence Data Analyses

As a result of RT-PCR tests, a unique AGVd isolate was determined in the area (cv. Menendi). AGVd-Turkish (AGVd-TR) isolate was molecularly characterized. For that purpose, the complete genome of the isolate was cloned and sequenced by using end to end genome specific primers. RNA genome of AGVd-TR isolate was aligned with the world isolates by using bioinformatic tools (Blastn, Vector NTI and CLC Main Workbench software) to construct a phylogenetic tree. In order to predict the most stable secondary structure of the AGVd-TR isolate, computer analysis was performed on the established full sequence.

RESULTS AND DISCUSSION

Results of RT-PCR Analysis

Although fifty five plant samples belonging to Barış Çekirdeksizi, Ergin Çekirdeksizi, Hamburg Misketi, Cardinal, Hafiz Ali, Yapıncak, Emir, Danam, İsa, Danuta, Ora, Calmeria, Menendi, Autumn Royal, Crimson Seedless, Siyah Korent, Kızlar Tahtası, Tekirdağ Çekirdeksizi, Uslu Üzümü and Sultani Çekirdeksizi cultivars were tested by RT-PCR, only one plant sample belonging to Menendi cultivar was found positive for AGVd. This isolate was named AGVd-TR isolate meaning AGVd isolate from Turkey.

Determination of Sequence Homology and Phylogenetic Analysis

In the present study, AGVd was isolated and characterized for the first time in Turkey. The complete genome of AGVd-TR isolate was determined and assigned to GenBank (accession no. KR706469). Based on phylogenetic analyses, AGVd-TR isolate showed a high degree of identity with the Indian (accession no. KJ019304), Chinese (EU743606) and Iranian (KF876037) isolates with a similarity of 97 %, 97 % and 96 % respectively. Similarity of different isolates was also showed phylogenetic tree (Figure 1).

Figure 1. Phylogenetic tree that showed identity of AGVd-TR isolate with AGVd world isolates.

As expected, the AGVd-TR isolate adopted a basic rod-like most stable secondary structure as reported for *Pospiviroidae* (Sanger et al. 1976; Ding and Owens, 2003) (Figure 2). In conclusion, primary and secondary structure analysis showed that the RNA genome of the AGVd-TR isolate differed from other compared isolates by only a few nucleotides in the way that deletion, insertion and substitution.

Figure 2. Secondary structure of AGVd-TR isolate

ACKNOWLEDGMENTS

This research was supported by the Scientific and Technological Research Council of Turkey (TUBİTAK) project TOVAG 113O209. Also, we thank our colleagues from Ege University, Yüzüncü Yıl University and Manisa Viticulture Research Station who provided insight and expertise that greatly assisted the research.

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PP 27 - Molecular characterization of *Grapevine yellow speckle viroid* 1 detected in the Aegean Region vineyards of Turkey

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INTRODUCTION

Grapevine yellow speckle viroid 1 (GYSVd-1) belonging to the genus *Apscaviroid* (*Pospiviroidae*) is one of the causal agents of Grapevine yellow speckle disease (Koltunow and Rezaian, 1988; Koltunow et al., 1989). Grapevine yellow speckle disease is appeared in the worldwide vineyards extensively and it causes vein banding, yellow speckle on leaves and stunting in grapevine plants (Randless, 2003). GYSVd-1 is one of the two viroids (GYSVd-1 and 2) which can induce symptoms in the grapevine and also widespread in the main vineyards of our country and region (Gazel and Önelge, 2003; Gökçek, 2007; Çopul, 2012).

MATERIALS AND METHODS

Plant Sample Collection and Reverse Transcriptase PCR Analyses

In the present study, forty nine grapevine leaf samples were collected from different vineyards from Aegean region. Collected plant samples belonging to different grapevine cultivars (Barış Çekirdeksizi, Hamburg Misketi, Hafız Ali, Calmeria, Yapıncak, Crimson Seedless, Danuta, Emir, Menendi, Ergin Çekirdeksizi, İsa, Uslu, Ora, Siyah Korent, Autumn Royal, Kızlar Tahtası, Sultani Çekirdeksizi) were tested by RT-PCR using GYSVd-1 spesific primers [forward 5' TTG AGG CCT GGC GTA ACG C 3' and reverse 5' GGA CGC GAA CGT GAA TAG G 3' (Koltunow and Rezaian, 1988)]. Also, RT-PCR analyses were carried out using specific primers suggested by Jiang et al. (2012) for GYSVd-3 (forward 5'-TTG GAT CCC ACC TCG GAA GGC CGC C-3' and reverse 5'-TTG GAT CCT AAC CAC AGG AAC CAC A-3').

Molecular Cloning and Sequence Data Analyses

For each grapevine cultivar, a GYSV-1 isolate from positive plant samples were selected randomly to perform molecular characterization. The complete genome of selected isolates (GYSVd-1-TR) were cloned and sequenced. RNA genomes of different GYSVd-1 Turkish isolates were analyzed by computer programs such as Blastn, Vector NTI and CLC Main Workbench. The sequences of Turkish isolates were aligned with other world isolates. Also, following the cloning and sequencing of complete genomes of GYSVd-3 positive isolates, the obtained results were then compared with the nucleotide sequences characterized by Jiang et al. (2012) (GenBank accession no. DQ371469 and DQ371470) using Vector NTI software. Finally, phylogenetic tree that showed identity of GYSVd-1 and GYSVd-3 Turkish isolates with world isolates was constructed using CLC Main Workbench computer software.

RESULTS AND DISCUSSION

RT-PCR Results and Positive Isolates for GYSVd-1 and 3

In consequence of RT-PCR analyses, twenty one of 49 plant samples (42,86%) belonging to Sultani Çekirdeksizi, Ora, İsa, Ergin Çekirdeksizi, Emir, Crimson Seedless and Calmeria cultivars were found positive for GYSvd-1. In RT-PCR analyses performed to determine GYSVd-3, twenty five of 49 plant samples (51,02%) belonging to different grapevine cultivars (Emir, Ergin Çekirdeksizi, Autumn Royal, Hafiz Ali, Ora, Danuta, Calmeria) were found positive.

Results of Phylogenetic Analyses

As a result of the alignments, the identity among different GYSVd-1 Turkish isolates varied between 79-96% (Table 1). Phylogenetic analyses revealed that GYSVd-1 Turkish isolates showed the highest identity with the isolates from Germany (GenBank accession no. X87906), USA (KF137564), Italy (EU682453), China (DQ371471), Australia (X06904), Hungary (GQ995473), India (AB742223), Iran (KF916046), Canada (AF462163), Thailand (AY639607) and Japan (AB028466) (Figure 1). Also, the identity among GYSVd-3 Turkish isolates and GYSVd-3 isolates characterized by Jiang et al. (2012) (GenBank accession no. DQ371469 and DQ371470) varied between 80-91% (Table 2).

GYSVd-1	Crimson S.	Emir	Ergin C.	Isa	Ora	Sultani C.
Calmeria	87	89	93	96	84	92
Crimson S.		84	87	90	82	91
Emir			89	91	79	87
Ergin C.				96	84	93
İsa					87	95
Ora						85

Table 1. Similarity table of molecularly characterized GYSVd-1 Turkish isolates selected from different grapevine cultivars (%).

GYSVd-3	DQ371469 and DQ371470
Emir	91
Ergin C.	90
Autumn R.	89
Hafiz Ali	89
Ora	86
Danuta	85
Calmeria	80

Table 2. Similarity table of molecularly characterized GYSVd-3 Turkish isolate and the isolates characterized by Jiang et al. (2012) (GenBank accession no. DQ371469 and DQ371470) (%).

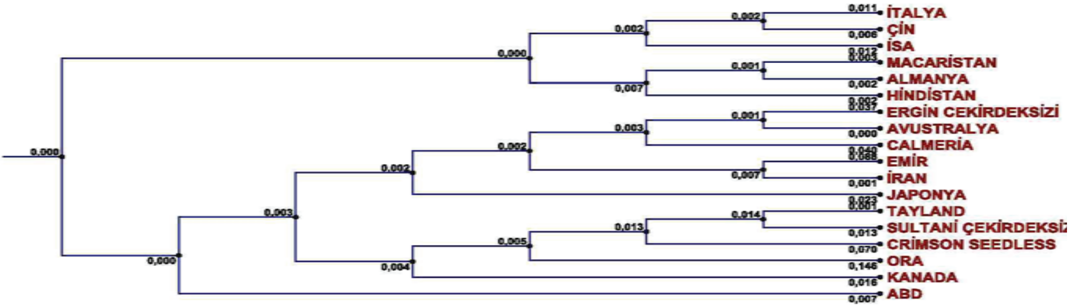

Figure 1. Phylogenetic tree that showed identity of GYSVd-Turkish isolates with GYSVd-1 world isolates.

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PP 28 - Survey of grapevine viruses in the east and southeast regions of Turkey

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INTRODUCTION

Turkey is one of the important countries for viticulture due to being of the center of grapevine gene pool covering Middle Asia-Mediterranean zone. Grape production in Turkey was 6.4% of world production in 2012 and 22% of grape production in Turkey was from The East and Southeast Regions of Turkey (Anonymous, 2013). The virus diseases are one of the limiting factors for viticulture via effecting the quality and quantity of the product. The objective of this study is to assess the current sanitary status in terms of virus diseases of commercial grapevine orchards in The East and Southeast Regions of Turkey.

MATERIALS AND METHODS

Field surveys were carried out in grapevine growing areas in the East and Southeast Regions of Turkey during spring and autumn 2014. In spring time, a total of 87 samples (out of 16 from Batman, 19 from Diyarbakır, 37 from Elazığ, 3 from Mardin, 12 from Şanlıurfa provinces), in autumn time, a total of 123 samples (out of 18 from Adıyaman, 9 from Batman, 23 from Elazığ, 42 from Malatya, 13 from Mardin, 18 from Şanlıurfa provinces) were collected from grapevines showing virus symptoms. Most of the collected varieties were local varieties such as Ağbesni, Islahiye Karası, Kabarcık, Boğazkere, Şire, Ağın, Öküzgözü, Köhnü, Mazruni, Tahannebi, Trakya and Ilkeren. All samples were individually tested by DAS-ELISA for the presence of *Grapevine leafroll associated virus -1* (GLRaV-1), *Grapevine leafroll associated virus -2* (GLRaV-2), *Grapevine leafroll associated virus -3* (GLRaV-3), *Grapevine leafroll associated virus -4* (GLRaV-4), *Grapevine leafroll associated virus -5* (GLRaV-5), *Grapevine leafroll associated virus -6* (GLRaV-6), *Grapevine leafroll associated virus -7* (GLRaV-7), *Grapevine leafroll associated virus -9* (GLRaV-9), *Grapevine fanleaf nepovirus* (GFLV), *Grapevine fleck virus* (GFkV), *Grapevine virus A* (GVA), *Raspberry ringspot nepovirus* (RpRSV), *Strawberry latent ringspot nepovirus* (SLRSV), *Tomato black ring nepovirus* (TBRV), *Arabis mosaic virus* (ArMV) and also by PCR for *Grapevine red blotch associated virus* (GRBaV) using the primer pairs designed in this study.

RESULTS AND DISCUSSION

Virus symptoms were observed in most of the visited vineyards. The symptoms were red foliage (on red varieties), short internodes, mottling of leaves, yellow mosaics or flecks on leaves, vein bandings, fasciation of canes, double internodes, excessive growth from secondary buds, straggly bunches with both large and small berries, rolling of the leaves in the fall, wood pitting and grooving. The overall infection level in all samples was 14.76%. The most prevalent virus was GFLV (6.66%) followed by GLRaV 4-9 (3.80%), GLRaV 1+3 (3.81%), GFkV (1.43%) and GVA (0.95 %). All samples were individually infected and no mix infection. RpRSV, SLRSV, TBRV, ArMV, GLRaV-2 were not detected among the all samples

PCR analysis of positive control DNA of GRBaV amplified the expected size of 1063 bp amplicon. GRBaV is a DNA virus and has a circular genome. The new primer pair provides amplification from the region of 3’ end and 5’ end of the GRBaV isolate. Blast analysis of the sequenced amplicon approved the validity of the GRBaV specific primer pair. PCR analysis of all 210 grapevine samples revealed negative results for GRBaV.

Through these researches, a deteriorated sanitary status for the local grapevine varieties was demonstrated. Also, newly identified virus disease of GRBaV in the world was investigated in Turkish vineyards.

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PP 29 - Survey on a new emergent grapevine disease and *Grapevine Pinot gris virus* (GPGV) in Veneto, Northeast Italy.

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INTRODUCTION

An emergent grapevine disease characterized by leaf mottling and deformation was identified for the first time on cv. Pinot gris in Northern Italy in 2003. The disease was later observed in other varieties, mainly in other Italian regions but also in other European countries, such as Slovenia. The most sensitive cultivars identified so far are Pinot gris, Pinot blanc, Pinot noir, Gewürztraminer, Tocai friulano and Glera. The disease was recently suggested to be associated to a newly discovered virus, named GPGV (*Grapevine Pinot gris virus*) (Giampietruzzi et al., 2012); however its aetiology is still not completely clear, as it was found both in symptomatic and asymptomatic plants. The aim of the present work was to survey the occurrence of the disease and of the virus in the Veneto region and to get some preliminary data on effect on vine production, with particular regards to Prosecco.

MATERIALS AND METHODS

In 2013-2014 a total of 222 vineyards, all cultivated with varieties known to be susceptible, were visually surveyed in the Veneto region (Northeast Italy) for the presence of the symptomatology. Most of them (172) were located in Treviso province. The majority of the vineyards were planted with cv. Glera (69,4%), another part with cv. Pinot gris (19,4%) and the remaining (11,2%) with other susceptible varieties. Vineyard age spanned from 2 to more than 50 years old. Symptomatic plants were identified and marked. PATCHY program (Maixner, 1993) was used to verify spatial clustering of diseased plants by runs-analysis and calculation of Morisita's index of dispersion.

In autumn 2014, the productive parameters were evaluated in 5 vineyards (3 from cv. Glera, 2 from cv. Pinot gris). Moreover, pH, sugar content and acidity of the must were determined. Statistical tests were performed with the IBM Statistical Package for Social Science (SPSS) program, using the analysis of variance (ANOVA).

Leaves were collected from at least one symptomatic and one asymptomatic grapevine per vineyard, and frozen at -80°C for molecular analyses. Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen) following MacKenzie et al. (1997) and converted into cDNAs, as described in Angelini et al. (2004). Detection of GPGV was performed by real time PCR assay using the newly designed primer pairs GPGV PolF1/R2, targeting the GPGV polymerase gene.

RESULTS AND DISCUSSION

Visual monitoring of symptoms in vineyard

Survey of the symptomatology in winegrowing areas of Veneto region showed that the disease occurs in many vineyards, especially in cvs. Glera and Pinot gris and in all the districts monitored. Among the 222 vineyards surveyed, 162 (73%) showed at least one plant with symptoms of the disease. The occurrence of symptomatic grapevines was generally not very high. Indeed, most vineyards (68.5%) exhibited a low occurrence of symptoms, less than 1%, while the remaining 42 showed a higher number of symptomatic plants, reaching in 9 cases more than 10%. No correlation was found between disease occurrence and vineyard age or other factors. Diverse spatial patterns can take place in vineyard: regular, random or clustered. In the most symptomatic vineyards, the statistical analyses of the disease pattern showed significant clustering of diseased grapevines.

Parameters at vintage

Disease impact on quality and quantity of grapevine production in cv. Glera seems less severe than in cv. Pinot gris. Concerning the grape total production per plant, very symptomatic plants of Pinot gris showed a strong reduction (80-85%) in both vineyards. Conversely, in Glera vineyards the decrease was approximately 66% in the first case and 35% in the second case, however in the third case these was no statistically significant reduction (Fig. 1). Also the average bunch

weight is significantly lower in the symptomatic plants, both in Pinot gris and Glera (Fig. 2). Other parameters, such as the number of bunches per plant and the average berry weight, did not show any important differences. These data generally confirmed previous results obtained in cv Pinot gris and Traminer in Trentino Alto Adige (Malossini et al., 2012). Concerning the qualitative parameters of the must, a higher acidity is shown by symptomatic plants in 3 cases out of 5. The pH and the sugar content did show significant differences only in some cases. Thus, the major effect of the disease seems to be a decrease of the total production per plant, due mainly to a reduced bunch size.

GPGV occurrence in Veneto vineyards

The presence of GPGV was assessed by real-time PCR in 298 samples taken from symptomatic (88) and asymptomatic (210) grapevines. Samples were collected in vineyards planted with susceptible varieties, regardless of whether or not they showed the disease. The virus occurred in all the samples collected from symptomatic grapevines (88 out of 88), while it was present in 150 out of 210 leaf samples taken from asymptomatic grapevines (71.4%). If only vineyards with higher symptomatology (more than 1% plants showing symptoms) are considered, the GPGV presence in asymptomatic samples reached 96.5%. Even in vineyards which did not show any plants with symptoms, the virus was widely present (56 infected grapevines out of 74).

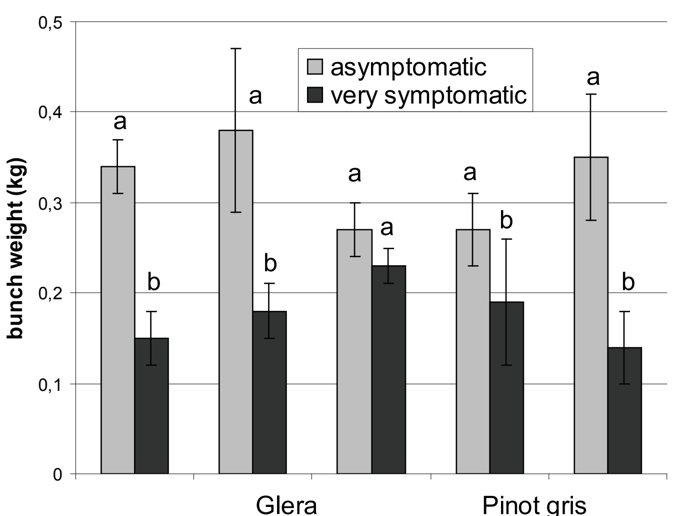

Figure 1. Vintage 2014: grape production per plant (kg) in asymptomatic and very symptomatic grapevines from 5 vineyards (3 cv Glera, 2 cv Pinot gris). For every value, the average of 3 plants and the standard deviation is reported. Different letters correspond to statistically-significant differences ($p < 0.05$).

Figure 2. Vintage 2014: bunch weight per plant (kg) in asymptomatic and very symptomatic grapevines from 5 vineyards (3 cv Glera, 2 cv Pinot gris). For every value, the average of 3 plants and the standard deviation is reported. Different letters correspond to statistically-significant differences ($p < 0.05$).

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PP 30 - Investigation of newly-emerging grapevine viruses in The Central Anatolia region of Turkey

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INTRODUCTION

The grapevines (*Vitis* spp.) have been cultivated in Europe and Asia for thousands of years and have a highly valuable agricultural and economic importance. As most of the vegetatively propagated crops, grapevines are exposed to the attacks of a variety of viral agents which play a major role, causing heavy economic losses (Martelli 2014). The improvements on the "high-through output" or "next generation" sequencing technologies allowed the discovery of several unknown viruses. The identification of the putative Marafivirus *Grapevine Syrah virus 1* (GSyV-1) is the first example of the application of this novel technology in grapevine virology (Al Rwahnih et al. 2009). *Grapevine vein clearing virus* (GVCV), is the first identified DNA virus in *Vitis* and it is associated with a disease called 'Grapevine vein clearing and vine decline syndrome' (Zhang et al. 2011). The second virus, *Grapevine Pinot gris virus* (GPGV), was originally identified in a cv. Pinot gris plant showing a syndrome characterized by leaf mottling and stunting (Giampetruzzi et al. 2011). Also, a new virus *Grapevine red blotch-associated virus* (GRBaV), were identified with NGS (Al Rwahnih et al. 2013). Aim of this study was to investigate these newly-emerging viruses in the Central Anatolia region of Turkey.

MATERIALS AND METHODS

In the summer of 2014, 200 grapevine samples which were showing virus-like symptoms were collected from Central Anatolian region of Turkey. The RNAs were extracted using a commercial kit (Qiagen, RNeasy Plant Mini Kit) and cDNA was synthesized using random primers with the Super Script Choice System (Invitrogen, USA). PCR analysis was performed with virus specific detection primers of GLRaV1, GPGV, GRBaV and GSyV (Alabi et al. 2011, Rwahnih et al. 2013, Glasa et al. 2014, Maliogka et al. 2015).

RESULTS AND DISCUSSION

Leafroll 1, which is the most common virus diseases of the grapevines were used for preliminary analysis. Based on the survey analysis from Central Anatolian samples, none of tested viruses were detected, except GLRaV1. These results show that the tested grapevines from Central Anatolian region of Turkey are not affected by these newly-emerging viruses, yet. Further studies are under investigation.

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PP 31 - *Grapevine yellow speckle viroid-3* (GYSVd-3), a tentative viroid species in Turkish vineyards

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INTRODUCTION

Grapevine is one of the most permissive, natural viroid hosts. Currently, five species of viroid; *Hop stunt viroid* (HpSVd) (Sano *et al*., 1986), *Citrus exocortis viroid* (CEVd) (Garcia-Arenal *et al*., 1987), *Australian grapevine viroid* (AGVd) (Rezaian, 1990; Guo *et al*., 2007; Elleuch *et al*., 2002), *Grapevine yellow speckle viroid-1* (GYSVd-1) (Koltunow and Rezaian, 1988), and *Grapevine yellow speckle viroid- 2* (GYSVd-2) (Koltunow and Rezaian, 1989), plus *Grapevine yellow speckle viroid-3* (GYSVd-3), tentatively classified into the genus *Apscaviroid* (Jiang *et al*., 2009). The viroid was first called as *Chinese grapevine viroid* (CGVd); however it was then proposed to name as GYSVd-3 based on the results of sequence and phylogenetic analysis. The viroid has the potential to form the rod-like secondary structure contains 366 nt. Preliminary results of laboratory assays showed the presence of GYSVd-3 in a small number of grapevine grown as outdoor plant. Therefore, this research was carried out to investigate the viroid in autochthonous grape varieties from Eastern Mediterranean and Southeast Anatolia vineyards in Turkey.

MATERIALS AND METHODS

Surveys were conducted in two grape regions, eastern Mediterranean and Southeast Anatolia in summer and green samples were collected to test with RT-PCR assays. Petioles were used to extract total nucleic acid with silica-capture method according to Foissac *et al.,* (2000) with slight modification. RT-PCR was performed with viroid-specific primers (Jiang *et al.,* 2009). Sequencing reactions were performed directly on RT-PCR products with primers corresponding to viral polarity by Genome Express (Grenoble, France) and sequences from Turkish isolates were compared with sequences retrieved from databanks. Nucleotide sequence aligments of the viroid isolates, were individually obtained by using CLUSTAL W program (Thompson *et al*., 1994). The MEGA v.05 software (Tamura *et al*., 2013) was used to estimate nucleotide diversity and for phylogeny construction and evaluation, using the neighbor-joining (NJ) method. The robustness of tree topology was evaluated with 1,000 bootstrap resamplings.

RESULTS AND DISCUSSION

A total of 351 samples from 67 vineyards in both regions was surveyed and fiftyfive samples produced GYSVd-3 DNA bands at 366 bp in size. Turkish sequence variants at phylogenetic tree showed a high percentage of nucleotide identity (approx. 98%) with the sequences of GYSVd-1, and contrarily low nucleotide identity with GYSVd-2 variants (≤ 90%). A phylogenetic analysis of 18 sequence variants from Eastern Mediterranean were grouped in six distinct branches and showed quite high identity with GYSVd-1 variants from Iran. Two sequences, one from Aegean and the other from eastern Mediterranean, out of all others showed were closely related to GYSVd-1 variants which had sequence homology with previously reported GYSVd-3 variants.

Consequently, there is significant variations among nucleotide sequences of GYSVd-1 and -3 and the effect of these viroids on biological properties is stil unknown. It is very important to find out the biological relationship between GYSVd and “yellow speckle” symptom in order to give final decision.

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PP 32 - Comparison of molecular hybridization, real time OCR and classical PCR techniques for diagnosis of GYSVD-1 (*Grapevine yellow speckle viroid-1*)

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INTRODUCTION

Viroids are the smallest known plant pathogens and have been identified as agents of several economically significant crop diseases. They comprise small (246– 401 nt), uncapsidated, covalently closed circular, non-coding RNA molecules which rely entirely on host factors for their replication (Flores et al. 2005; Ding 2009; Zhang et al. 2011).*Grapevine yellow speckle viroid* 1 (GYVSd-1) is a viroid in the family *Pospiviroidae*. GYSVd-1 is found in the important production areas, is very easily spread mechanically by contaminated cutting tools (Szychowski *et al*., 1988), infected graft (Szychowski *et al*., 1988; Staub *et al*., 1995), propagation materials (Kultonow *et al*., 1988) and causes severe damage to quality and quantity of grapevines. Rapid diagnosis of viroid infections may be obtained by bioassays, polyacrylamide gel electrophoresis (PAGE), molecular techniques such as polymerase chain reaction (PCR) and hybridization assays. In this study we aimed to compare three molecular methods including molecular hybridization, real-time pcr and classical pcr in order to detect GYSVd-1.

MATERIALS AND METHODS

Fifty plant specimens which are potentially positive for GYSVd-1 according to previous studies (Copul, 2012) were obtained from major grapevine production areas in Aegean region in 2014. RNA extraction was carried out according to the new-combined method as described in Paylan et al. (2014). To verify the amount of RNA, total RNA was quantified by image analysis of the ethidium bromide–stained RNA gel. The TNA obtained from this method, were used for complementary DNA synthesis via reverse transcription according to the First Strand cDNA Synthesis Kit Protocol. Molecular Hybridization studies were processed and applied according to procedure described by Guner et al., (2011) and probes were supported from Çandar (2014). The reverse transcriptase classical PCR assay was carried out as described by Copul (2012). Real-Time PCR assays were performed with Roche Real-Time PCR system under appropriate PCR conditions for GYSVd-1. cDNA mixture were amplified with FastStart Essential DNA Green Master (2x) (Roche, Germany) which was included Sybr Green I dye. At the end of the last PCR cycle, melting analyses were performed for eliminating non-specific products like primer dimers (Onder and Gumus, 2014). Water and negative controls were used in every stage of molecular assays.

RESULTS AND DISCUSSION

GYSVd-1 was detected in 45 of 50 samples by one or more detection techniques. Molecular hybridization had the lowest sensitivity, with only 20/45 GYSVd-1-positive samples were detected using this process. 37/45 positive samples were detected with classical PCR. Using Real-time PCR 44/45 positive samples were detected (Figure 1). 18 positive samples were detected in all methods. Three molecular methods for detecting GYSVd-1 compared in this work and we conclude that Real time PCR was the most sensitive amplification method followed by classical PCR and Molecular Hybridization. Also Real-time PCR method is less time consuming than classical PCR and hybridization methods. The presence of Taq DNA polymerase inhibitors in plant tissues may limit Molecular hybridization method’s usefulness for these samples. These results confirm the necessity of developing a more sensitive hybridization method. Both PCR methods appear to be important for the diagnosis of GYSVd-1. Real time PCR has proven to be a very sensitive method for the diagnosis of GYSVd-1 infection. The method is efficient, fast and practical, providing potential for use in routine diagnostic laboratories.

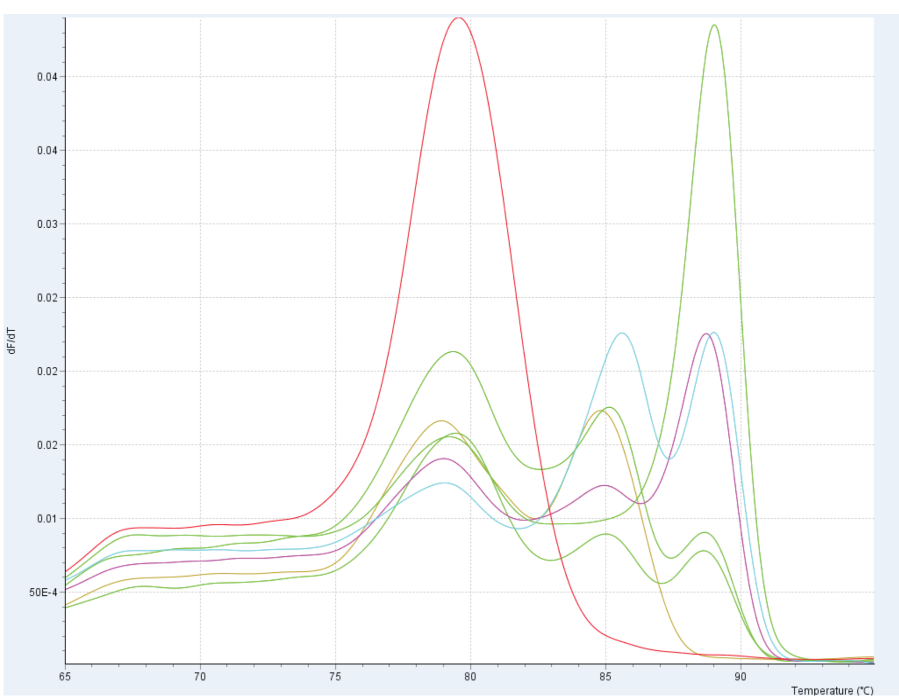

Figure 1. Real Time PCR for GYSVd-1; The melting curves of PCR products are shown.

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PP 33 - Symptom alterations in Australian grapevine viroid chimeras

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INTRODUCTION

Despite their small size, viroids have a complex genome in which the mechanism of pathogenicity and symptom induction remains unclear (Owens and Hammond, 2009). Species of the family *Pospiviroidae* have a rod-like secondary structure with five structural–functional domains: P (pathogenicity), C (central), V (variable), TL (terminal left) and TR (terminal right) (Keese and Symons, 1985).

Complex interactions between structural domains are determinant of symptom development and pathogenicity in *Pospiviroidae.* Studies with artificial chimeras derived from the viroids(Góra *et al.,* 1996; Visvader and Symons, 1986) have provided information on the relationship between specific regions of the viroid molecule and symptom expression particularly in the members of the genus *Pospivirod* (Gora *et al*., 1996; Visvader and Symons, 1996; Sano *et al*., 1992). Members of the genus *Apscaviroid* are restricted to woody plants and because of this, , studies on the relationship between their RNA secondary structural domains and their biological properties are very limited. *Australian grapevine viroid* (AGVd), a member of the genus *Apscaviroid,* is symptomless in grapevine, but induces stunting and mottling on tomato plants.*Grapevine yellow speckle viroid 1* (GYSVd1), on the other hand, induces yellow speckle symptoms in developed leaves of grapevine under favorable conditions. It is naturally restricted to grapevine (Hadidi *et al*., 2003).

The aim of this study was to evaluate the effect of exchanging distinct domains of AGVd with their corresponding parts from GYSVd1on symptom expression and identifying the pathogenicity determinants in a member of the genus *Apscaviroid*.

MATERIALS AND METHODS

The compositions of four chimeric molecules designed to exchange the structural domains of the secondary structure of AGVd and GYSVd1 are shown in Fig. 1A., The segments were replaced so to preserve the CCR and the secondary structure of AGVd. Constructs designated AGYSd-TL, AGYSd-P, AGYSd-V and AGYSd-TR contained replacements of TL, P, V, or TR, respectively, from GYSVinto the corresponding regions of AGVd genome. The constructs were put under control of the 35s promoter and agroinoculated to tomato and cucumber plants. Monomeric AGVd and GYSVd1 DNA in the same vector were inoculated as positive and negative controls, respectively. Three weeks post-inoculation, RNA was extracted from newly grown leaves of inoculated plants and RT-PCR was carried out using AGV-H/C primer pair corresponding to CCR domain of AGVd (Wan Chow Wah and Symons, 1997).

RESULTS

RT-PCR analysis and amplicon sequencing confirmed production of *de novo* populations of AGVd-GYSVd 1 chimeras in the inoculated plants. This meant that, all chimeras could replicate in the inoculated plants. Sequencing of PCR fragments from infected plants showed that the resulting progeny is identical to the original sequence. GYSYd1 did not replicate in the inoculated plants.

Symptoms of AGVd wild type in tomato plants were stunting, mottling and leaflet deformation. But, tomato plants inoculated with AGYS-TL showed rugosity, severe leaf deformation, leaf curl and severe narrowing of apical leaves; AGYS-P induced rugosity, leaf curl and mild yellowing on new leaves of tomato. AGYS-V caused severe deformation and twisting of leaves in infected plants. AGYS-TR developed only stunting and mottling (Fig. 1B). All chimeric molecules were similar to AGVd wild type in inducing stunting in tomato. Stunting was the only symptom generated by replication of the chimeras in cucumber plants.

DISCUSSION

The mechanisms of symptom induction by viroids are poorly understood (Owens and Hammond 2009).In the present study, replacement of the TL and P domains of AGVd by those of GYSVd1 altered the severity of symptoms in tomato. But

exchange of V domain led to change in the symptom types. TR domain had no obvious effect on the symptoms. Symptom development and pathogenicity are complex aspects of viroid biology. Disease is not generated only by a single domain of the viroids. Rather, interactions of different viroid domains as well as interaction of the latter with host factors play important roles in disease development (Gomez *et al.* 2008).

In chimera construct of the genus *Pospiviroid* severity of symptoms is determined by TL and P domains. However V and TR domains also interact with those domains in symptom induction (Góra *et al*., 1996, Owens and Hammond 2009).

In PSTVd, severity of symptoms and symptom type are highly dependent on the concentration of the viroid generated siRNA in the tissues (Góra *et al*., 1996; Owens and Hammond ,2009; Sano *et al*., 1992). Enhancement of symptom severity by the replacement of the TL and P domains may be due to changes in affinity of the viroid RNA for host factors (Itaya *et al.*, 2002), or provide the generation of certain new specific viroid-derived siRNAs (Carrington and Ambros, 2003).

Figure 1. A, Schematic representation of AGVd-GYSVd 1 chimeras used in the present study. Hatched areas represent parts of GYSVd 1 in corresponding regions of AGVd genome **. B,:**Symptoms induced in tomato by AGVd (mottling), AGYS-TL (severe leaflet deformation) AGYS-P (rugosity and mild yellowing), AGYS-V (twisting and severe malformation of leaves), and AGYS-TR (mottling and leaf curl) compared to healthy control (H).

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PP 34 - *Hop stunt viroid,* a good candidate for internal control in detection of viroids and viruses in grapevine

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INTRODUCTION

Six viroids are reported to infect grapevine from the family *Pospiviroidae* including *Hop stunt viroid* (HSVd), *Grapevine yellow speckle* 1 and 2 (GYSVd1 and GYSVd2), *Australian grapevine viroid* (AGVd), *Citrus exocortis viroid* (CEVd) (Flores *et al*., 2005) and *Grapevine latent viroid* (GLVd) (Zhang *et al*., 2014). To detect these RNA genome agents of grapevine, inclusion of an internal control assay is recommended to remove the possibility of PCR false negatives due to extraction failure, nucleic acid degradation or presence of PCR inhibitors. Previously housekeeping genes have been used as the internal controls but these genes need to treated with DNase when performing diagnostic test. Here, we suggest *Hop stunt viroid* as the reliable internal control in detecting viroids and viruses in grapevine (*Vitis vinifera*) by RT-PCR assay.

MATERIALES AND METHODS

To gain an insight into presence of HSVd in grapevine, during 2011-213, 154 samples were collected from North-West (West-Azerbaijan, East-Azerbaijan and Ardabil Provinces) and West (Kurdistan Province) of Iran.Total nucleic acid was extracted from leaves by the silica-capture method (Foissac*et al*., 2000) and used as template for reverse transcription polymerase chain reaction (RT-PCR). Reverse transcription was done with random hexamer primers and PCR with the forward (HSVd-78P: 5′-AACCCGGGGCAACTCTTCTC-3′) and reverse (HSVd-83M: 5′-AACCCGGGGCTCCTTTCTCA-3′) primers corresponding to nucleotides 76-95 and 85- 66 of HSVd, respectively, (Sano *et al*., 2001) using *Pfu* DNA polymerase. The RT-PCR products were purified from agarose gel using the “Quantum PrepTM Freeze □N Squeeze DNA Gel Extraction Spin Columns” (Bio Rad, USA), then ligated into the pGEM-T Easy vector (Promega, USA) and used for transformation of competent *Escherchia coli* DH5α cells. The recombinant plasmids were sequenced with the M13 F and M13 R universal primers using an automated DNA sequencer (MWG operon, Germany).

RESULTS AND DISCUSSION

RT-PCR showed that all of the assayed 154 Iranian vines were infected by HSVd (Figure 1). On the other hand, high incidence of HSVd in Japan (88%) (Sano *et al*., 1986), Czech Republic (70%) (Matousek*et al*., 2003) have also been recorded. Therefore, it seems that this viroid is widespread in many places of the world. When six variants, collectively from the three Iranian isolates, were sequenced it was revealed that they are 297 nt in lenght. Five variants had nucleotide sequence identical to each other and to that of variant (accession No. E01844) retrieved from GenBank. The variant HSVd-Bm10.2 (accession No. KF97099) (Hajizadeh *et al*., 2015), derived from a vine in Maragheh (East-Azerbaijan), showed just one mutation (G_{67} to A_{67}) with respect to the grapevine HSVd reference strain (accession number: E01844). In phylogenetic analysis based on full length HSVd sequences, the six Iranian isolates and previously reported GenBank accessions recovered from grapevine formed a cluster with HSVd variants commonly recovered from hop. This data confirm a close relationship between HSVd-hope and HSVd-grape variants (data not shown) (Sano *et al*., 2001).

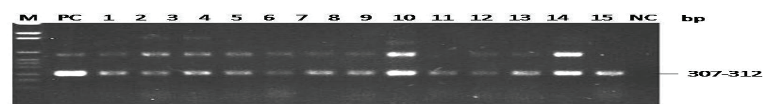

Figure 1. Agarose gel electrophoresis analysis of DNA fragments amplified by RT-PCR with a HSVd-specific primer pair. Lane M, DNA marker VI (Roche); lane PC, Positive control; lanes 1 to 15, samples from vineyard in different location with symptoms similar to that of HSVd; lane NC, healthy control.

Due to the widespread prevalence of HSVd in most grapevine cultivars (100% in the west and northwestern region of Iran), we propose to use this viroid as an internal control in surveying the vine for presence of viroids and viruses in grapevine by RT-PCR. An advantage of this choice is that HSVd shares many structural characteristics with the other grapevine viroids and, contrary to internal controls derived from host mRNAs or rRNAs (Gambino *et al*., 2009), no DNase treatment is needed prior to reverse transcription. This saves time and costs when performing the diagnostic tests. For the same reasons, HSVd could be a feasible internal control for multiplex RT-PCR protocols for detection of viroids (Hajizadeh *et al*., 2012) and RNA viruses in grapevines as well.

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PP 35 - Spread of GPGV-associated disease in two vineyards in Trentino (Italy)

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INTRODUCTION

Symptoms of chlorotic mottling, stunting and leaf deformation in cultivars Pinot gris and Traminer were reported in Trentino since 2003. In 2011, a new virus, named *Grapevine Pinot Gris Virus* (GPGV) was identified by deep sequencing and shown to be likely associated with the disease (Giampetruzzi *et al.*, 2012). Subsequently, GPGV was detected in Friuli Venezia Giulia, Veneto, Emilia Romagna, Lombardia and Apulia and, outside of Italy, in Slovakia and Cezch Republic (Glasa *et al.*, 2014), Slovenia (Mavric Plesko *et al.,* 2014), France (Beuve *et al.*, 2015), Greece and South Korea (Cho *et al.*, 2013). In this latter country symptoms being partially different for they recalled the necrosis of berries observed in vines infected with the related trichovirus *Grapevine berry inner necrosis virus* (GINV).

The present work analyzed the spread of the GPGV-associated disease in two vineyards in Trentino on the cvs P. gris and Traminer, during seven and six years, respectively.

MATERIALS AND METHODS

Vineyards

Location: Zablani (Mezzocorona, Trento)

This vineyard was planted in 2003 and 2005, with the cv. P. gris grafted onto SO4 rootstocks. The plot contains 1053 vines and is trained with the Guyot training system. All vines were inspected for the presence of symptoms of leaf mottling and deformation every year between 2009 and 2015 in May and June .

In addition, 10 symptomatic (A) and 10 asymptomatic (N) vines were tested by RT-PCR every year from 2011 to 2014 for the presence of GPGV and in 2012, 2013 and 2014 for the presence of viruses regulated in the Italian certification system [*i.e. Grapevine virus A* (GVA)*, Grapevine virus B* (GVB)*, Grapevine leafroll-associated virus 1, 2, 3* (GLRaV-1, -2, -3)*, Grapevine fanleaf virus* (GFLV)*, Grapevine fleck virus* (GFkV) *and Arabis mosaic virus* (ArMV)].

Location: Coveli (Faedo, Trento)

This vineyard was planted in 2003, with the cv. Traminer grafted onto different rootstocks. The plot contains 1106 vines and is trained with the "pergola Trentina" training system. All vines were inspected for the presence of symptoms of leaf mottling and deformation every year between 2010 and 2015 in May and June. In addition, 8 symptomatic (A) and 9 asymptomatic (N) vines were tested for the presence of GPGV and the Italian-regulated viruses as above described.

Survey for disease and virus detection:

Symptoms were ranked on a three-scale, mild, medium and high, according to their severity and extent of canopy involved. Total RNA extraction, cDNA synthesis and RT-PCR for Italian-regulated viruses, were done according to Faggioli *et al.* (2012). GPGV was detected by RT-PCR according to Giampetruzzi *et al.* (2012) and Saldarelli *et al.* (2015).

RESULTS AND DISCUSSION

The spatial distribution of vines showing symptoms during the observed periods shows a similar progress in both vineyards consisting in an initial active expansion, which reaches a plateau. Particularly, the incidence of symptomatic grapevines in the cv. P. gris and Traminer vineyards, increases from 13.3% to 33.9% and from 2.7% to 6.78% during the first 4 and 5 years of observation, respectively. After this initial increase percentages of vines showing symptoms did not increase further and remain stable around these values resulting in 2015 percentages of diseased vines of 34.5% and 6.3% in P. gris and Traminer vineyards, repectively. Furthermore, the spatial distribution of symptomatic grapevines showed an aggregated pattern, suggesting a slow vine-to-vine spread within single rows.

RT-PCR assays showed that GPGV was present in all initial 10 symptomatic and 10 symptomless P. gris vines but, throughout the 7 years of observations, 4 out the 10 symptomless vines started showing symptoms of variable severity. Similar assays in selected cv Traminer vines detected GPGV in all 8 symptomatic and 4 out of 9 symptomless plants. However, among these 4 initially symptomless vines, two GPGV-infected plants, started to display symptoms throughout the time of observation.

These new symptomatic vines occurred, in the P. gris vineyard, close to the existing diseased vines whereas, in the cv Traminer vineyard their appearance was random. Besides, all vines were free of all the Italian regulated-viruses.

The present study suggests that GPGV was initially introduced in the two vineyards with infected plant material for diseased vines occur in aggregated spots. The existence of a slow-moving putative vector in GPGV transmission cannot be excluded since newly diseased vines emerged close to existing ones. Appearance of symptoms in GPGV-infected but initially symptomless vines is likely explained by a shift or superinfection of viral variants from symptomless to symptomatic as suggested by Saldarelli *et al.* (2015) and Bianchi *et al.* (2015).

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PP 36 - Molecular variability of grapevine virus D isolates from naturally infected vineyards in Tunisia

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INTRODUCTION

Rugose wood (RW) is a complex disease affecting grapevine worldwide. It is latent in ungrafted *Vitis vinifera* and American hybrid rootstocks, but expresses its symptoms in grafted vines (Martelli and Boudon Padieu, 2006). Grafting onto woody indicators allows to distinguish at least four distinct viral syndromes, i.e. stem pitting on *V. rupestris*, stem grooving on Kober 5BB, corky bark and stem grooving on LN33. Six different viruses belonging to the family *Betaflexiviridae,* five of which of the genus *Trichovirus* [*Grapevine virus* A (GVA), *Grapevine virus* B (GVB), *Grapevine virus* D (GVD), Grapevine virus E (GVE) and Grapevine virus F (GVF)] and one of the genus *Foveavirus* [*Grapevine rupestris stem pitting-associated* virus (GRSPaV)] have been associated to the disease (Martelli, 2014). Previous studies showed that GVA, GVB, GVD and GRSPaV are very common in the Tunisian vineyards (Mahfoudhi *et al*., 1998; 2014; Soltani *et al*., 2013). For some of those viruses, in particular GVA, GVB and GRSPaV, the existence of a wide variability between isolates of the same species has been demonstrated through comparative molecular analyses that can give reason of the different symptoms on vines (Borgo et al., 2009; Goszczynski et al., 2008, Goszczynski, 2010; Murolo et al., 2008; Shi et al., 2004), while scanty remains the information available for GVD, both in terms of genetic variability and etiological role in RW disease. To better explore the incidence and the molecular features of the Tunisian GVD isolates, a survey has been carried out in the Tunisian vineyards and in a grapevine germplasm collection of the National Institute of Agronomic Research of Tunisia (INRAT).

MATERIALS AND METHODS

Wine and table grape samples (284 in total) analyzed in this study were from 15 commercial vineyards (207 samples) and a germplasm collection of local varieties (77 samples) at INRAT.

Total nucleic acids (TNAs) were extracted from *ca.* 100 mg of leaf vein tissues, homogenized in 1ml of grinding buffer (4.0M guanidine isothiocyanate, 0.2M NaOAc pH 5.2, 25mM EDTA, 1.0M KOAc pH 5.0 and 2.5% (w\v) PVP-40), then purified using silica particles as described by Foissac *et al*. (2001). The first strand cDNA was synthesized using 1 µg of TNA extracts mixed with 0.5 µg of random hexamer primers and 200 units *Moloney murine leukaemia virus* (M-MLV) reverse transcriptase, in a final volume of 20 µl.

All samples were tested by RT-PCR for the presence of GVD using the specific primers CP471C and CP7V (Abou Ghanem *et al*., 1997) that amplify a 474 bp fragment from the coat protein gene (CP). All amplicons were analyzed by Single Stranded Conformation Polymorphism (SSCP) assay, performed directly to cDNA-generated PCRs of the positive samples, according to Martins-Lopes et al. (2001).

Direct sequencing was done with the same primers used for RT-PCR and made upon DNA from the representative isolates for each SSCP pattern observed. CLUSTALX was used to generate the multiple sequence alignments (Thompson et al., 1997). The phylogenetic tree was constructed by using NEIGHBOR, SEQBOOT, PROTDIST and CONSENSE programmes of the PHYLIP package (Felsenstein, 1989).

RESULTS AND DISCUSSION

RT-PCR assays successfully amplified the expected 474 bp product from 112 out of 284 tested samples (39.4%). GVD was present in 9 commercial vineyards with different levels of infection and in 27 vines (out of 77) of the collection plot, in line with the infection rate of 41.4% previously reported by Mahfoudhi *et al*. (2014). SSCP analysis of PCR amplicons from positive isolates showed the existence of 11 different electrophoretic profiles.

Accordingly, one sample for each SSCP profile (7 samples from commercial vineyards and 4 from the collection plot) was sequenced. Nucleotides sequence analyses showed that the Tunisian GVD isolates shared among them 79-98%

identity, with GVD-T11 being the most variable isolate (Table 1).

In the phylogenetic analysis GVD-T4 and GVD-T10 isolates shared 91% nucleotide identity between them and clustered in a same group with the Brazilian isolates Garg and Dolc (Fig. 1). The isolates GVD-T6, GVD-T7, GVD-T8, GVD-T9 and GVD-T14 clustered together in a distinct group close to the Italian isolate Ab.Gh, showing 93-98% nucleotide identity. Finally, GVD-T12, GVD-T13 and GVD-T15 isolates clustered in another clade. Interesting was the dislocation in the tree of GVD-11 isolate, far from all the other GVD isolates, with which it shared 79-84% identity at the nucleotide level.

This study expands the knowledge on the incidence of GVD in the Tunisian vineyards and provides the first molecular information on the presence of an high sequence variability that merits a further genome exploration.

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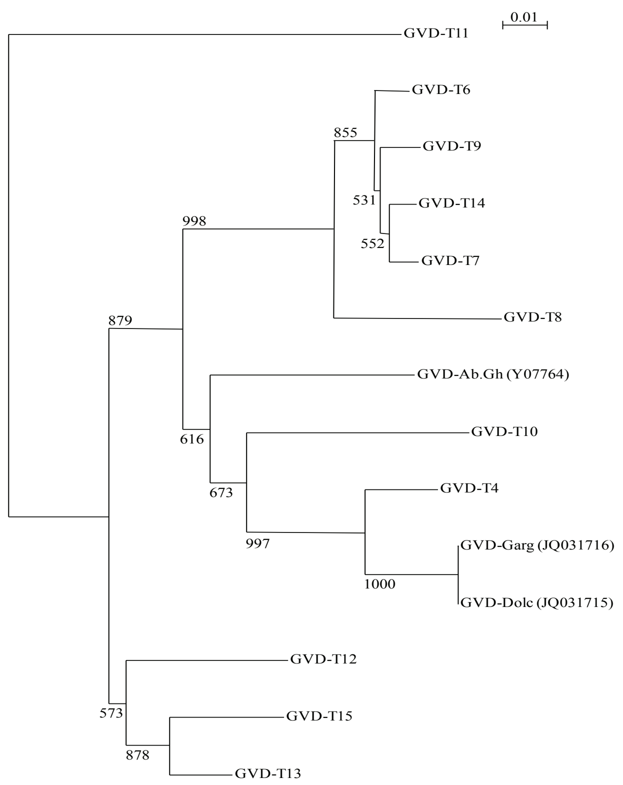

Figure 1: Phylogenetic tree constructed with the coat protein sequences (nts) of GVD isolates from Tunisia (T4, T6-15) and homologue from different origins present in Genbank. Numbers on branches indicate percentage of support out of 1000 bootstrap replications.

PP 37 - Preliminary data on the transmission of *Grapevine Pinot Gris virus* by *Colomerus vitis*

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INTRODUCTION

Grapevine Pinot Gris Virus (GPGV), a member of *Trichovirus* (*Betaflexiviridae*), was first identified in Trentino vineyards (Giampetruzzi et al., 2012) and later recorded in other Italian districts (Emilia-Romagna, Veneto, Friuli-Venezia Giulia, Apulia and Lombardy) as well as South Korea, Slovenia, the Czech and Slovak Republics (Saldarelli et al., 2014), Greece (Martelli, 2014) and France (Beuve et al., 2015). The occurrence of GPGV was associated to leaf deformation, chlorotic mottling and stunting, but the virus was also recorded in symptomless vines (Giampetruzzi et al., 2012). The associated disease, consisting in symptoms of leaf mottling and deformation, causes economic losses due to the reduction in number, weight and quality of berries which may not be compatible with vine production (Malossini et al., 2012) The transmission mechanisms of GPGV are still unclear. GPGV was transmitted by grafting to *Vitis riparia* Michx. and *V. vinifera* L. (Saldarelli et al., 2013 and 2014) and attempts to transmit GPGV mechanically to herbaceous plants (*Nicotiana occidentalis* H.-M. Wheeler and *Chenopodium quinoa* Willd.) were unsuccessful (Beber, 2012). Furthermore, a putative vector has not yet been identified. Genomic sequence analysis demonstrated a close phylogenetic relationship between GPGV and *Grapevine berry inner necrosis virus* (GINV) (Giampetruzzi et al., 2012; Yoshikawa et al., 1997) which was considered to be vectored by the eriophyid mite *Colomerus vitis* (Pagenstecher), based on field trials (Kunugi et al., 2000). The aim of this work was to assess the presence of GPGV on *C. vitis* collected from GPGV-infected and symptomatic grapevines and to verify if this mite can transmit the GPGV to healthy grapevine plants.

MATERIALS AND METHOD

Specimens of *C. vitis* were collected in GPGV symptomatic vineyards from north-eastern Italy and mites were extracted from infested buds and leaf erinea using the washing method described in Monfreda et al. (2007). GPGV infection of vines from which mites were collected was ascertained using RT-PCR (Saldarelli et al., 2014). An aliquot of each collected mite sample was subjected to morphological species identification. To assess the presence of GPGV in mites, pools of 5-15 *C. vitis* individuals were placed alive in Eppendorf tubes at + 4-5°C for about 24 h to allow digestion of ingested food and then stored at -80°C before RNA extraction. Two different methods were used to extract total RNA from different numbers of individual eriophyid mites (5-15 specimens): the Qiagen RNA Micro kit and the Trizol Reagent. Complementary DNA (cDNA) was obtained from total RNA after random-primed reverse transcription using M-MLV reverse transcriptase. PCR reaction was performed on cDNA, with specific primer pairs as described in Saldarelli et al. (2014). The PCR products were cleaned using EXOSAP, sequenced and compared with those present in the GenBank (Acc. Num. NC 015782). Transmission trials were carried out under controlled condition (22°C, 70% U.R., 16:8 L:D) using *C. vitis* infested buds and leaf erinea collected from GPGV infected vines. Mites were placed onto healthy grapevines of cv Pinot Gris (34 plants) and Traminer (11 plants) and analysed to assess the presence of GPGV before and after transmission trials.

RESULTS AND DISCUSSION

Morphological studies confirmed that the eriophyid mites collected from leaf erinea and overwintering in buds belonged to the species *C. vitis.* The leaf symptoms associated with the mite are those typically induced by the “erineum” strain. Based on the data of Carew et al. (2004) it was not possible to establish the presence of individuals of the bud strain.

Preliminary assays using total RNA purified with column- (Qiagen) or phenol-based (Trizol) protocols failed to detect GPGV in groups of 5-10 mites, probably because of the limited amount of purified total RNAs. The addition of glycogen as a carrier for total RNA precipitation allowed GPGV detection using RT-PCR. Confirmation of the obtained amplicons was verified by direct sequencing. In RT-PCR a strong correlation between the number of mites and the intensity of the GPGV amplified bands was observed, although the virus was not detected in all mite samples.

The data obtained in this work showed that GPGV is present in the body of *C. vitis* collected from the population infesting the buds and leaf erinea of virus-infected grapevine plants, even after one day of starving during which the mites were preserved at low temperatures. In addition, the presence of bleach and detergent should remove viral particles present on the exposed mite surface, including mouthparts and distal parts of related preoral chambers.

Data obtained from the analysis carried out on plants after the transmission trials showed the presence of GPGV in 7 out of 34 vines of cv Traminer and 1 out of 11 grapevine plants of cv Pinot Gris. Results obtained from transmission trials suggest that *C. vitis* may acquire GPGV and transfer the virus to healthy grapevines, being a potential candidate vector for natural GPGV transmission. Other eriophyid mites are vectors of the trichoviruses *Peach mosaic virus* (PcMV) (Gispert et al., 1998) and *Cherry mottle leaf virus* (James & Mukerji, 1993). In the case of PcMV, a semi-persistent mode of transmission was demonstrated (Gispert et al., 1998), whereas eriophyid involvement in GINV transmission was demonstrated by field and greenhouse spread trials and by the overlapping distribution of *C. vitis* and the virus in the field (Kunugi et al., 2000). Further investigations are needed to assess whether *C. vitis* could be a real vector for GPGV.

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PP 38 - Detection of GFLV, ArMV, GLRaV-1 and GLRaV-3 in a single tube real-time PCR based on melting curve analysis with EvaGreen®

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INTRODUCTION

Based on EU Directive 2002/11/EC, mother stock plant material for vegetative propagation should not contain *Grapevine fanleaf virus* (GFLV), *Arabis mosaic virus* (ArMV), *Grapevine leafroll-associated virus 1* (GLRaV-1) and *Grapevine leafroll-associated virus 3* (GLRaV-3). Bioassays and serology have been widely used for the detection of grapevine viruses, but more recently, nucleic acid-based methods started to be widely applied (Pacifico et al., 2009). Traditional gel-based PCR technology for the simultaneous detection of several viruses in a single tube is only possible by working with different fragment sizes (Gambino, 2015; Xu et al., 2012). This technique, however, is both time consuming and has the risk of potential carry-over contamination (Barletta et al., 2013). Due to the absence of post-amplification procedures, multiplex real-time PCR allows rapid analysis with a larger sample throughput (Garrido et al., 2012) and limits carry-over. A method based on TaqMan® probe real time multiplex RT-PCR was developed to detect grapevine viruses regulated by the EU Directive 2002/11/EC (Lòpez-Fabuel et al. 2013). However, when assessing the specificity of the reaction using DNA intercalating dye, melting curve analysis is performed after each PCR run. The different peaks of this melting curve may distinguish multiple pathogens amplified in a sample (Khan et al., 2011; Cheng et al., 2013; Rao et al., 2014). Each peak is influenced by the length, GC/AT ratio and sequence of the fragment (Wehrle et al., 2010). Aim of this work was to develop a sensitive, fast and easy-to-use multiplex PCR with melting curve analysis, based on EvaGreen® intercalating agent, to evaluate the presence/absence of GFLV, ArMV, GLRaV-1 and GLRaV-3 in grapevines.

MATERIALS AND METHODS

The virus genomic sequences used in this study were derived from GenBank database. For GLRaV-3, ArMV and GFLV, the known coat protein sequences were aligned by Geneious software (Biomatters Ltd., New Zealand) in order to design primers targeting short sequences (87-222 bp) in conserved regions; for GLRaV-1, the heat shock protein (HSP-70) sequences were used. In addition, primer sets were selected to generate amplicons with distinct melting temperatures, so able to separate the different virus targets during the melting curve analysis. The amplicon melting temperatures were predicted by uMELT v.2.0.2, using Blake & Delcourt algorithm (Dwight et al., 2011).

Plasmids containing target sequences were used to verify experimentally the different amplicon melting temperatures and to study threshold limit. The analytical specificity was tested amplifying each plasmid with multiplex primer mixture. Multiplex PCRs were done in 20 µL, including 10 µl of SsoFast EvaGreen Supermix (Bio-Rad Laboratories, USA), 4 µl of a pool of 5 µM primers (GLRaV-3: ArMV: GFLV: GLRaV-1 = 1,25:0,75:1:1), 1.5 µl of each plasmid DNA (approximately 1 to 2.5 ng). The cycling program was: 95°C for 5 min, then 40 cycles at 95° for 15'', 60° for 30''. The melting curve protocol was automatically selected by SDS software v2. 3 (Thermo Fisher Scientific, USA). All the real time PCR reactions were run on an 7900ht platform (Applied Biosystems, USA).

To preliminarily assess the specificity and diagnostic capacity of the method, seven single- or multi-infected plant samples, previously tested by ELISA, along with two healthy grapevines, were sampled as dormant canes. Total RNAs were extracted from phloem scrapings by silica capture (Foissac et al., 2005). A random primed cDNA was synthesized using MMLV-RT (Thermo Fisher Scientific, USA) from an average amount of 500 ng RNA for the amplification step.

RESULTS AND DISCUSSION

In positive samples, different peaks corresponding to each virus, were generated after melting curve analysis (Fig. 1). The melting temperature (Tm) corresponding to GLRaV-3 peak was ≈78°C, to ArMV ≈79.5, to GFLV ≈82,2 and to GLRaV-1 ≈84, respectively (Table 1). In negative samples (healthy and no template controls), no peaks having target virus Tm were observed. The virus detection in each plant sample, by the melting curve in multiplex real-time PCR, substantially confirmed ELISA results (Table 1). By contrast, in C3 the molecular method recognized a peak of 84.5°C corresponding to GLRaV-1, while in C4 peaks of 78°C and 84.7°C were identified, disclosing the presence of GLRaV-3 and GLRaV-1, respectively, which previously escaped serological detection. Therefore, this multiplex amplification, coupled with the melting curve analysis, proved to be a fast and unbiased tool for an improved screening of regulated grapevine viruses.

Sample	GLRaV-3		ArMV		GFLV		GLRaV-1	
	Mux-PCR	ELISA	Mux-PCR	ELISA	Mux-PCR	ELISA	Mux-PCR	ELISA
C1	78	+	-	-	-	-	-	-
C2	-	-	-	-	82.5	+	-	-
C3	78	+	-	-	82	+	84.6[a]	-
C4	78.4[a]	-	-	-	82	+	84.7[a]	-
C5	78	+	-	-	82	+	84.5	+
C6	78	+	-	-	82	+	84.5	+
C7	77.8	+	-	-	83	+	84.5	+
Healthy 1 *V. vinifera*	-	-	-	-	-	-	-	-
Healthy 2 seedling	-	-	-	-	-	-	-	-
GLRaV-3 plasmid	77.3		-		-		-	
ARMV plasmid	-		79.5		-		-	
GFLV plasmid	-		-		82.1		-	
GLRAV-1 plasmid	-		-		-		83.7	

Table. 1. Detection of the four EU-regulated viruses by multiplex PCR (Mux-PCR) melting curve analysis versus ELISA. Temperature (°C) of melting curves is indicated for each amplicon. [a] Presence of a specific melting peak revealing the potential virus infection, which was not detected by ELISA. GFLV: *Grapevine fanleaf virus*, ArMv: *Arabis mosaic virus*, GLRaV-1: *Grapevine leafroll-associated virus 1* and GLRaV-3: *Grapevine leafroll-associated virus 3*.

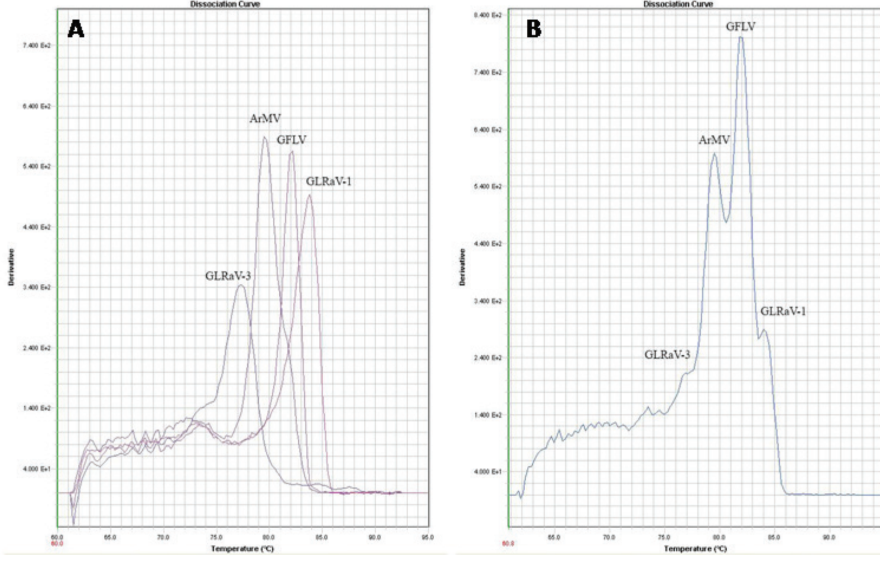

Figure 1. Melting curve analysis showing specificity of the EvaGreen® multiplex real-time PCR assay. (A) Mixture of four distinct reactions amplifying each virus plasmid. (B) Single reaction with a pool of four plasmids amplified in multiplex. Dissociation curves are plotted as negative derivative of fluorescence over temperature (y axis) versus temperature (x axis).

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PP 39 - Grapevine viruses in the United Kingdom

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INTRODUCTION

More viruses have been identified in grapevines than in any other woody perennial crop, at least 65 different viruses belonging to nearly 30 different genera. Diseases caused by grapevine viruses can be classified into four principal groups: leafroll, degeneration/decline, rugose wood and fleck. These diseases have a worldwide distribution and have been reported from most grape-growing countries. There has been limited surveillance for viruses affecting grapevine in the UK and therefore a survey was initiated to ascertain the presence of viruses in the country.

MATERIALS AND METHODS

A screening programme was undertaken in 2014 to establish the viral status of the approximately 100 grapevine (mainly *Vitis vinifera*) cultivars grown by the Royal Horticultural Society in Wisley. These cultivars have been collected since the 1970s and include table and wine grapes grown in the field and under glass. Vines were inspected for symptoms every fortnight from June to September 2014. In July inter-veinal reddening and downward rolling of leaf margins developed on mature leaves of the red-fruited cultivar ‘Queen of Esther’. Yellowing and downward rolling of leaf margins developed from August on mature leaves of the white-fruited cultivar ‘Theresa’. Both cultivars were observed to have swelling above the graft union. Samples of leaves and petioles were collected in August 2014 from the two symptomatic cultivars and a further six randomly selected cultivars. Cambium scrapings from dormant canes of all eight cultivars were taken in late September 2014.

Samples of leaves and petioles were collected from eight cultivars selected at random from the 39 grapevine (*V. labrusca* and *V. vinifera*) cultivars in the National Fruit Collection in Faversham, Kent.

RNA was extracted using an RNeasy Plant Mini Kit (Qiagen, Manchester, UK) and tested using specific primers for nine grapevine viruses in multiplex RT-PCR as described by Gambino and Gribaudo (2006).

RESULTS AND DISCUSSION

Amplicons of the expected size were obtained using primers for *Grapevine virus A* (GVA), *Grapevine fleck virus* (GFkV) and *Grapevine rupestris stem pitting-associated virus* (GRSPaV) using leaf and petiole samples from the two symptomatic cultivars (‘Queen of Esther’ and ‘Theresa’). GRSPaV was also detected in ‘Nero’ and ‘Seyval Blanc’ in Wisley, and in ‘Dunkelfelder’, ‘Faberrebe’, ‘Madeleine Angevine’, ‘Regner’ and ‘Siegerrebe’ in the National Fruit Collection. The same results were obtained using samples from dormant canes and in addition *Grapevine leafroll-associated virus 1* (GLRaV-1) was also detected in ‘Queen of Esther’. Amplicons from ‘Queen of Esther’ were directly sequenced from both directions and the sequences deposited in GenBank (Accession Nos KP238179, KP151488, KP238178 and KP284454). The amplicons obtained using primers for GVA, GFkV, GLRaV-1 and had 93, 100, 98 and 100% nucleotide sequence identity with Accession Nos. HQ671651 (GVA, China), JN133945, (GFkV, Slovakia) JF811857 (GLRaV-1, USA) and JQ922417 (GRSPaV, USA), respectively.

These are the first reports of GVA, GFkV, GLRaV-1 and GRSPaV in the UK. GVA is genetically variable; some variants are asymptomatic whilst others are associated with rugose wood, a disease of grafted vines characterized by pits and grooves on the stem of the scion and/or rootstock. One variant is associated with Shiraz disease which causes decline and eventual death of affected vines (Martelli, 2014). GFkV has limited economic impact on commercial plantings but may cause disease in *V. rupestris* or vines grafted onto such rootstocks (Martelli, 2014). GLRaV-1 is one of five distinct viruses that cause grapevine leafroll, one of the most damaging viral diseases of grapevines (Naidu *et al*., 2014). GRSPaV causes rupestris stem pitting of grapevines and has a broad distribution worldwide. The disease is normally of limited consequence and does not cause specific leaf or stem symptoms on most cultivars (Zhang *et al*., 1998). However, in some rootstocks derived from *V. rupestris* parentage (e.g. ‘St. George’) the stem becomes covered with small pits and grooves which may expand to encompass the whole cylinder. Vines grafted onto such rootstocks have reduced vigour. All four viruses are transmitted via vegetative propagation. GVA and GLRaV-1 are also transmitted by mealybugs (Pseudococcidae) and scale

insects (Coccidae) (Naidu *et al*. 2014). Not all of these vectors are known to be present in the UK and some are restricted to protected environments (e.g. *Pseudococcus longispinus*), but *Parthenolecanium corni* and *Pulvinaria vitis* are widespread in both protected and unprotected environments.

These are the first reports of viruses infecting grapevines in the UK. There seems to have been limited monitoring or testing for viruses in UK vineyards and therefore it is difficult to speculate on the national distribution of these viruses. As part of our study we visited commercial vineyards in Suffolk and Surrey, and grapevines in these locations did not show symptoms of virus infection.

The UK's grapevine industry is developing rapidly and in 2013 the planted area was estimated to be 1,884 hectares, compared with 1,215 hectares in 2009 (English Wine Producers, 2014). Pathogens such as GVA and GLRaV-1 have the potential to reduce grapevine yield and quality, and restrict cultivar choice. Education is vital to increase awareness of the impacts of these diseases and to ensure the use of certified planting stock and the adoption of best management practices, to advance the sustainability and profitability of the UK industry.

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PP 40 - Survey of emerging viruses in Switzerland

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INTRODUCTION

In recent years, next-generation sequencing (NGS) has significantly improved our understanding of viral pathogens. The NGS approach has allowed the identification of several new viruses. For most of these newly described pathogens, there is still little information available about their spread and prevalence in commercial vineyards. Therefore, we conducted a study to monitor the occurrence of three emerging viruses in Swiss vineyards: *Grapevine red blotch-associated virus* (GRBaV), Grapevine redglobe virus (GRGV) and *Grapevine Pinot gris-associated virus* (GPGaV). GRBaV, a tentative member of the family *Geminivirideae*, is a recently discovered viral pathogen associated with the red blotch disease (Al Rwahnih *et al.*, 2013). GRBaV has not yet been reported outside North America. Grapevine redglobe virus (GRGV) belongs to the family *Tymoviridae* and was first described in southern Italy (Sabanadzovic *et al.*, 2000). GRGV was detected later on in Greece, California and France. *Grapevine Pinot gris-associated virus* (GPGaV), a trichovirus, was first identified on Pinot gris plants showing leaf mottling (Giampetruzzi *et al.*, 2012). Glasa *et al.* (2014) also identified GPGaV, yet did not observe an association between GPGaV and any specific symptom. Recently, Saldarelli *et al.* (2015) showed that different GPGaV lineages possibly had different biological properties: some isolates were associated with symptoms, and others were not.

MATERIALS AND METHODS

The vineyards in the La Côte region cover a surface of *ca.* 2000 ha and are located on the edge of Lake Geneva, between Lausanne and Geneva. Fifty commercial vineyards were randomly selected in the La Côte appellation. Vineyards were at least ten years old. Within each vineyard, a plot (500m^2) was defined and 20 individual grapevines were sampled at random. Samples, consisting in dormant canes, were collected in January 2012. To account for the possible uneven distribution of the virus within a vine, three dormant canes per plant were collected. All samples collected from one location were then bulked for nucleic acid extraction, using RNeasy Plant Mini Kits (Qiagen). GPGaV and GRGV infection were assessed by RT-PCR and GRBaV by PCR. One-step reverse transcription-polymerase chain reaction was performed with the AMV reverse transcriptase (Promega, Germany), GoTaq polymerase (Promega, Germany) and total RNA as template. The primers used in this study were as follows: CPfor/ CPrev for detecting GRBaV (Krenz *et al.*, 2014); DetF/DetR for GPGaV (Saldarelli *et al.*, 2015) and RG-CF-F1/ R1 for GRGV (Beuve *et al.*, 2015). To confirm viral infection, amplicons were cloned into pGEM-T easy vector (Promega, Germany) and sequenced at Fasteris SA (Switzerland). Nucleotide alignments were created using ClustalW. The phylogenetic relationships were determined using the software MEGA (version 6). Phylogenetic trees were generated using the maximum likelihood algorithm with 1000 bootstrap replicates.

RESULTS AND DISCUSSION

GRBaV was not found during this survey, whereas GRGV was frequently detected in grapevines in the vast majority of studied locations (83 %). Preliminary observations showed no specific symptoms associated with this virus. Further work will be necessary to clarify the effect of GRGV infection in grapevine.

GPGaV was found in seven locations (*i.e.* prevalence of 15%). The resulting 598 bp amplicons were sequenced in order to evaluate the phylogenetic relationship within GPGaV isolates. Three sequences obtained from our viral collection were added, and the 10 sequences were labeled GPGaV_CH1 to 10.These Swiss isolates are all closely related, the maximum genetic variability being only 6 % in the MP/CP region. The low heterogeneity of GPGaV was also reported for Slovak and Italian isolates. When these sequences were compared with publicly available ones, the identity score ranged from 94 to 99%. According to phylogenetic analyses, Swiss isolates segregated into two clades (Figure 1). All, except one, Swiss isolates clustered with French and Slovak isolates. The exception, GPGaV_CH1, grouped into a different clade, along with symptomatic isolates described by Saldarelli *et al.* (2015). C/T polymorphism in the MP stop codon was observed among Swiss isolates (Figure 2), as previously mentioned by others (Glasa *et al.*, 2014; Saldarelli *et al.*, 2015). So far, all GPGaV isolates identified in Switzerland were found on cultivar Chasselas and no specific symptoms were noted. Biological indexing has been initiated to evaluate if these different isolates can induce leaf mottling symptoms, when inoculated on cultivar Pinot gris. Further studies are clearly needed to evaluate the impact of GPGaV on grape and on wine production.

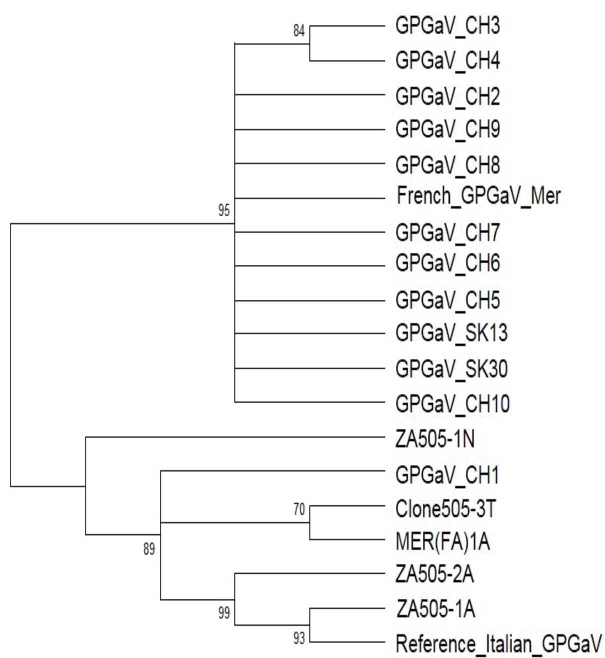

Figure 1. Maximum likelihood phylogenetic tree using nucleotide sequences of MP/CP genes present in 10 swiss GPGaV isolates and 9 other published isolates (*e.g.* French_ GPGaV_Mer = KM491305; reference_ Italian_ GPGaV = NC_015782). Branches are condensed and the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test is shown next to the branches.

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French_GPGaV_"Mer"         TGAGCGAGGCGAATCAAGTACTTCATGGGTTGACAGAAGGCAACAAAGAT 450
GPGaV_CH7                  TGAGCGAGGCGAATCAAGTACTTCATGGGTTGACAGAAGGCAACAAAGAT 450
GPGaV_CH6                  TGAGCGAGGCGAATCAAGTACTTCATGGGTTGACAGAAGGCAACAAAGAT 450
GPGaV_SK13                 TGAGCGAGGCGAATCAAGTACTTCATGGGTTGACAGAAGGCAACAAAGAT 450
GPGaV_SK30                 TGAGCGRGGCGAATCAAGTACTTCATGGGTTGACAGAAAACAACAAAGAT 450
GPGaV_CH5                  TGAGCGAGGCGAATCAAGTACTTCATGGGTTGACAGAAGGCAACAAAGAT 450
GPGaV_CH10                 TGAGCGAGGCGAATCAAGTACTTCATGGGTTGACAGAAGGCAACAAAGAT 450
ZA505-1N                   TGAGCGAGGCGAATCAAGTACTTCATGGGTTGACAGAAGGCAACAAAGAT 450
Clone505-3T                TGAGCGAGGCGAATCAAGTACTTCATGGGCTGACAGAAGGTAACAAAGAT 450
MER_FA_1A                  TGAGCGAGGCGAATCAAGTACTTCATGGGCTGACAGAAGGTAACAAAGAT 450
ZA505-1A                   TGAGCGAGGCGAATCAAGTACTTCATGGGCTGACACAAGGTAACAAAGAT 450
Reference_Italian_GPGaV    TGAGCGAGGCGAATCAAGTACTTCATGGGCTGACACAAGGTAACAAAGAT 450
ZA505-2A                   TGAGCGAGGCGAATCAAGTACTTCATGGGCTGACAGAAGGTAACAAAGAT 450
GPGaV_CH1                  TGAGCGAGGCGAATCAAGTACTTCATGGGCTGGCAGAAGGTAACAAAGAT 450
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PP 41 - Distribution and genetic diversity of Grapevine viruses in British Columbia

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INTRODUCTION

British Columbia (BC) is the second largest grape-growing region in Canada with the majority of production concentrated in the Okanagan Valley. Viruses and virus-like agents are considered significant constraints for grapevine (*Vitis vinifera* L.) production worldwide. Among them, Grapevine leafroll disease (GLRD), associated with Grapevine leafroll associated viruses (GLRaVs), is considered to be the most wide-spread disease of wine-grapes affecting vine growth and fruit quality. Although a survey was conducted in the major grape-growing regions of Canada during the mid-90s (MacKenzie et al., 1996), which recorded the occurrence of *Arabis mosaic virus* (ArMV), *Grapevine fanleaf virus* (GFLV), Grapevine leafroll associated viruse-1 (GLRaV-1) and Grapevine leafroll associated virus-3 (GLRaV-3) in BC vineyards, there is no current information available on the sanitary status of grapevines with an emphasis on viruses infecting grapevines. Therefore, we have undertaken large-scale surveys to record the incidence of major grapevine viruses viz., GLRaVs (GLRaV-1, -2, -3 and 4-9), GFLV, *Grapevine fleck virus* (GFkV), ArMV and *Grapevine red blotch-associated virus* (GRBaV). Furthermore, the genetic diversity and the evolutionary relationship of GLRaV-2 and GLRaV-3 isolates based on partial heat-shock protein (Hsp-70h) gene were determined for representative virus isolates and compared with reported global isolates.

MATERIALS AND METHODS

Field surveys were carried out during the 2013 and 2014 grape growing seasons and a total of 1,957 random-composite (5 vines per composite sample) and 293 target-individual grapevine samples from 10 red and 12 white cultivars from 113 vineyard blocks were collected in different wine-growing regions of BC. Leaves from random composite samples were tested for the presence of GLRaV-1, -2 -3 and 4-9, GFLV, GFkV, ArMV by DAS-ELISA using commercial kits (BIOREBA). Presence of GRBaV was tested by PCR assay following conditions described either by Al Rawhnih et al. 2013 or by Poojari et al., 2014. RT-PCR was carried out for GLRaVs, GFLV, ArMV and GFkV following the protocol described by Poojari et al., 2014. The RT-PCR amplicons were purified using QIAquick PCR purification kit (QIAGEN) and cloned into pTOPO2.1 (Invitrogen) following manufacturer's protocol. Two clones of partial Hsp-70h gene of GLRaV-2 and GLRaV-3 were sequenced from both directions. Sequence analysis and phylogenetic relationships were inferred using MEGA6 and SDT v1.2 (Tamura et al., 2013 and Muhire et al., 2014).

RESULTS AND DISCUSSION

Among the GLRaVs tested by ELISA, the most widespread was GLRaV-3 (17.2%), followed by GLRaV-2 (5.5%), GLRaV 4-9 (4.2%) and GLRaV-1 (1.4%). Low incidence of GFLV (0.5%) was detected from a total of 998 composite samples, whereas GFkV was detected at a much higher incidence (29.2%) from 788 composite samples (Figure 1). Two positives were detected for GRBaV from a total of 539 composite and 195 targeted samples tested using PCR. No positives were detected for ArMV from a total of 998 composite samples. RT-PCR analysis of representative samples confirmed the presence of the above mentioned viruses occurring as single and/or mixed infections. Nucleotide sequence analysis of partial Hsp-70h gene of 11 GLRaV-2 isolates showed high level of identity (90.0 to 99.3%) among BC isolates. Phylogenetic analysis of GLRaV-2 isolates with global GLRaV-2 variants belonging to six (H4, 93/955, PN, PV20, BD and RG), showed that GLRaV-2 BC isolates grouped with PN (Pacific Northwest) lineage, indicating the presence of a single variant of GLRaV-2 in BC vineyards. Sequence analysis of partial Hsp-70h gene of 15 GLRaV-3 isolates showed nucleotide sequence identities in the range of 88.7 to 99.8 % belonging to two distinct clades in comparison with the representative GLRaV-3 global isolates.

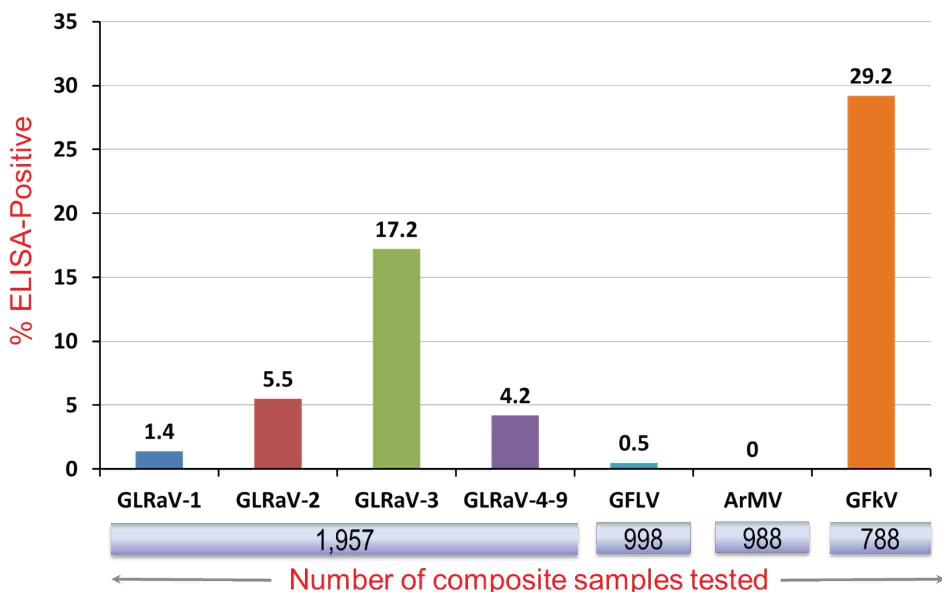

Figure 1: Incidence of grapevine viruses detected by DAS-ELISA in British Columbia vineyards

The high level of GLRD incidence and distribution in BC vineyards underlines the importance of insect vector (mealybugs and scale insects) management as well as planting clean plant material. Studies on insect vector diversity, population dynamics and their role in the disease etiology are currently underway. The information generated in the present study would serve as a new paradigm for understanding the epidemiology of major grapevine virus diseases and to design management strategies to control grapevine diseases in BC vineyards.

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PP 42 - A highly effective technology for isolation of RNAs from grapevine leaves throughout the season for use in virus diagnostics

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INTRODUCTION

Grapevines are infected by 64 distinct species of viruses that belong to diverse taxonomic groups (Martelli et al., 2012). Nucleic acid-based diagnostics, such as PCR, RT-PCR, RT-qPCR and deep sequencing are the most powerful technologies for virus diagnosis and discovery and constitute an integral component of the clean stock certification programs for grape production (López et al., 2009). The success of molecular diagnostics and discovery of viruses is contingent upon quality RNAs. However, isolation of highly pure RNA from grapevine plants, especially the old leaves, has been very challenging and problematic (Iandolino et al., 2004; Krenz 2014). This is due to the presence of high levels of secondary metabolites, such as polyphenols and polysaccharides in grapevines. Numerous methods have been developed to isolate total RNA from grapevines (Gambino et al., 2008; Iandolino et al., 2004; Japelaghi et al., 2011; Tattersall et al., 2005). However, these protocols take hours or even days to carry out, require extraction with hazardous chemicals, and involve many steps which could result in problems with cross contamination in virus detection. Furthermore, isolated RNAs often contain high levels of inhibiting substances that impair downstream amplification, leading to unreliable test results. Several RNA isolation kits have been developed by biotech companies and used for isolation of RNAs from diverse plant species, mostly annual herbaceous plants. These kits all use a silica-based column, involve a simple and straightforward procedure, avoid the use of organic solvents and yield quality RNAs within a short period of time. Some of the kits have been attempted for isolating RNA from woody plants with various degrees of success (Abdullahi, 2011; Tzarfati et al., 2013). However, a systematic comparison of the effectiveness of these commercial kits for the isolation of nucleic acids from grapevine tissues has not been conducted.

The objectives of this study were to compare and refine RNA isolation kits for use in isolation of large quantities of pure RNAs from grapevines. We have also made significant improvement to the RNA isolation methodology, so that it is suitable for the detection of both RNA and DNA viruses in grapevine throughout the entire growing season

MATERIALS AND METHODS

Young and mature leaves were collected from *Vitis vinifera* var. Chardonnay from growth chamber in the University of Guelph. Leaves were also collected from 6 varieties of *V. vinifera* (Chardonnay, Riesling, Syrah, Cabernet Franc, Gamay, and Gewurztraminer) from June to November from vineyards in Niagara, Ontario. Five commercial RNA isolation kits were selected for this study, which included TRIzol Reagent (Life Technologies), RNeasy Plant mini kit (Qiagen), Spectrum™ Plant Total RNA Kit (Sigma), AccuPrep viral RNA extraction kit (Bioneer) and Plant/fungi total RNA kit (Norgen Biotek). Total RNAs were isolated from leaves following the instruction of each kit. The quality and quantity of the RNA preparations were assessed with a NanoDrop spectrophotometer, electrophoresis on 1.5% formaldehyde–agarose gels, Bioanalyzer (Agilent) and RT-PCR or RT-qPCR detection of *Grapevine rupestris stem pitting-associated virus* (GRSPaV), the most widespread virus in grapevines (Meng and Gonsalves, 2007).

RESULTS AND DISCUSSION

Comparison of five commercial kits in isolating RNA from grapevines

Five of the most commonly used commercial RNA isolation kits were selected and compared for their effectiveness in isolation of RNA from both young and mature leaves of grapevine (*V. vinifera* cv. Chardonnay) maintained in a growth chamber. The results showed that the Spectrum plant total RNA kit from Sigma gave the best RNA yield of 39.9 µg from young leaves, followed by kits from Norgen and Bioneer (Fig.1). The Sigma, Norgen and Bioneer kits all produced high quality RNAs as indicated with an over 2.0 of A260/A280 (Fig. 1) and with a RIN of 9.0, 8.9 and 7.7, respectively. As expected, all the three kits produced less amounts of RNA from mature leaves compared to young leaves (Fig. 1), but the best is still the Sigma kit, followed by Bioneer and Norgen. Regardless of either young or mature leaves were used, Qiagen's kit failed to isolate RNA (Fig. 1). Similarly, Trizol® reagent also failed to isolate RNA from the grape leaves due to the insolubility of the pellet containing RNA (Fig. 1).

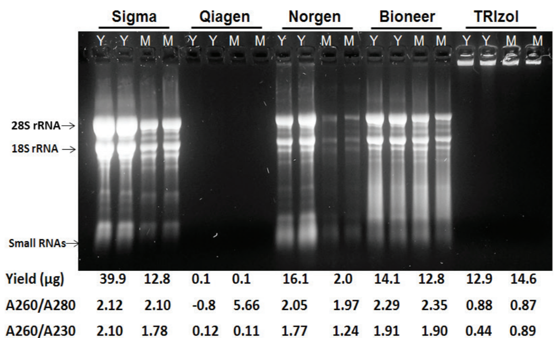

Figure 1. Denaturing gel electrophoresis of total RNA isolated from grapevine leaves. 50 mg of young (indicated as Y) and mature (M) leaves was used in RNA isolation with the five kits. The total RNA yield (µg), A260/A280 and A260/A230 ratios averaged from two replicates are given below the gel panel. 28S rRNA, 18S rRNA and small RNAs are indicated with arrows.

It was also shown that Norgen's kit outperformed the other four kits in terms of low molecular weight RNAs (Fig. 1). The results from Agilent Bioanalyzer analysis confirmed that Norgen kit produced the highest yield of small RNA (4.15 *µg*), followed by Bioneer's (1.15 *µg*).

RT-qPCR with primers targeting GRSPaV capsid protein gene confirmed that the RNAs isolated with Sigma, Norgen and Bioneer kit were qualitatively and quantitatively satisfactory in the detection of GRSPaV in grapevine as indicated with the low quantitation cycle (Cq) value of 21.6, 24.8 and 24.2, respectively. The results also showed that the Cq value of Sigma kit was about 3 (for capsid protein gene) and 1 (for ubiquitin) cycles lower than those from Norgen and Bioneer kit, which suggest that Sigma kit produced better quality RNA with less inhibitors than those of Norgen and Bioneer.

Inclusion of 2.5% PVP-40 in the lysis buffer is critical for isolating RNA from old leaves of grapevines

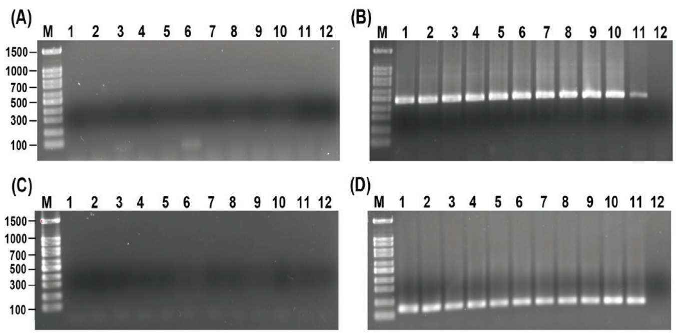

Figure 2. RT-PCR detection of GRSPaV (A and B) and ubiquitin (C and D) from RNAs isolated from grapevine leaves with Spectrum™ Plant Total RNA Kit (Sigma) with standard method (A and C) and modified method (B and D) (see Table 1). M: molecular size marker (bp); lane 12: water control.

As shown above, the Sigma kit is the best performer in isolating total RNA from young and mature leaves of grapevine from growth chamber, its effectiveness in isolating total RNA from grapevine leaves from vineyards was then tested. The results showed that the kit could isolate total RNA with high yield and good quality from leaves collected in June. However, it was not effective for isolating RNAs from symptomatic leaves collected in September. As shown in Table 1, the standard Sigma kit produced total RNA with very low yield (2.4 µg on average) and very low quality, as indicated with an average A260/A280 of 1.03 and A260/A230 of 0.32. As expected, the resulting RNAs were not suitable for the detection of GRSPaV and the reference gene, ubiquitin, using RT-PCR (Fig. 2A and 2C). The total RNAs isolated with Norgen kit from these samples were also not usable (not shown). Efforts were made to tackle this problem and we found that modification of the Sigma kit by adding 2.5% PVP-40 in the lysis buffer dramatically improved its performance in isolating RNA from old and symptomatic leaves collected in late summer and the fall. The modified method increased the total RNA yield to 10.0 µg on average, with high quality as judged by A260/A280 values of 2.04 and 1.96 of A260/A230. RT-PCR results showed that GRSPaV and ubiquitin were readily detected from all these RNA samples (Fig. 2B and 2D). Addition of 2.5% PVP-40 in the lysis buffer of the Norgen kit, however, did not improve its performance in isolation of total RNA from these old leaf samples (data not shown).

Table 1. Total RNA isolated from old leaves of grapevines with standard or modified protocol based on Sigma kit. 50 mg of old and diseased leaves of *Vitis vinifera* Cabernet Franc was used in RNA isolation with standard or modified protocol where 2.5% of PVP-40 being added in the lysis solution.

Leaf Sample	RNA yield (µg)		A260/280		A260/230	
	Standard	Modified	Standard	Modified	Standard	Modified
CF-1	3.1	8.8	1.08	2.08	0.29	2.00
CF-2	1.2	8.5	1.49	2.06	0.48	2.03
CF-3	2.1	8.6	1.69	2.06	0.64	2.06
CF-4	1.0	5.7	0.71	2.14	0.11	1.56
CF-5	0.5	5.9	0.90	2.06	0.13	1.90
CF-6	0.6	13.1	1.08	2.03	0.21	2.11
CF-7	1.8	8.1	0.86	2.03	0.18	1.95
CF-8	4.8	9.5	0.94	1.99	0.57	1.85
CF-9	4.6	12.4	0.95	1.99	0.38	2.03
CF-10	1.4	13.0	0.78	2.10	0.16	1.94
CF-11	4.8	16.8	0.81	1.87	0.42	2.17
Average	2.4	10.0	1.03	2.04	0.32	1.96

The effectiveness of the modified method based on Sigma kit in isolating total RNA from old grape leaves were further validated by testing over a hundred old leaf samples from both red and white varieties for eleven viruses. The results showed that the total RNAs isolated using the modified Sigma procedure are of sufficient quality, and are suitable for RT-PCR to detect diverse RNA viruses as well as for PCR to detect DNA virus *Grapevine red blotch-associated virus*. We have also shown that leaf tissue can serve as a reliable source throughout the entire growing season for the detection of viruses with the use of the improved RNA isolation technology. The improved methodology would receive broad utilizations in research on grapevines and many woody perennials, including the diagnosis and discovery of viruses and viroids.

ACKNOWLEDGEMENTS

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PP 43 - Flashdiag® FD: an innovative field diagnostic kit based on isothermal amplification for detection of Flavescence Dorée in *Vitis vinifera*

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INTRODUCTION

The European wine sector is facing a severe epidemic yellowing disease caused by Flavescence dorée phytoplasma (FDP). As no curative treatment exists, prophylactic control consists in uprooting the diseased plants and insecticide treatments against the insect vector *Scaphoideus titanus.* Early detection of symptomatic vines is essential to avoid the development of extensive outbreaks. Nucleic acid extracts from symptomatic plants are currently submitted to PCR/qPCR assays performed in expert laboratories. The emergence of new molecular technologies, like isothermal nucleic acid amplification, could represent an improvement in the management of grapevine yellowing diseases. Isothermal amplification targeting FDP specific gene applied to an appropriate sample of crude vine extract allows the detection of the corresponding amplicons with the Flashdiag® FD within less than one hour. The aim of the present work was to compare specificity and sensitivity of Flashdiag®FD kits in comparison to the French diagnostic protocol based on a triplex Real Time PCR method.

MATERIALS AND METHODS

Forty-one symptomatic grapevine samples for yellow diseases were used in this study. Total nucleic acid was extracted from 1g of petiole with the official DNA extraction protocol (LNPV, 2010). Concentration of total nucleic acid concentrations was measured by UV spectrometry. *Catharanthus roseus* periwinkles infected with several phytoplasmas and healthy periwinkles coming from INRA Bordeaux collection were used in order to test the specificity of Flashdiag®FD kits. Periwinkle DNA extracts were performed according to previous publication (Arnaud et al., 2007).

Flashdiag®FD kits are used after adding a rehydration solution to the lyophilized pellet, containing all isothermal amplification components. Then, 1 µl of total nucleic acid extract is used for each kit. The reaction is heated at 39°C for 20 min and amplification products are directly placed in a detection chamber with the immunological lateral flow strip. If there is one control line (upper line), the result is negative and if there is one control line and one test line (lowest line), the result is positive (see picture 1, below).

One line: no detection

Two lines: detection of FD

Picture 1: Flashdiag®FD results with lateral flow device

Taqman Real Time triplex PCR is performed following the official protocol: Detection of vineyard phytoplasmas of 16SrV group (Flavescence Dorée) and 16SrXII group (Bois Noir) triplex real time PCR (LNPV, 2010 adapted from Pelletier et al., 2009).

RESULTS

Out of the 41 infected samples, 29 reveal FD infection with the real time triplex PCR method. Out of those 29 samples, 28 were FD positive with the Flashdiag®FD method. So, a preliminary sensitivity of the Flashdiag® FD test is estimated at 96, 6**%** (*ie* the probability of a positive result is obtained when the disease is present) (See Figure 1).Out of the 12 remaining samples that revealed no FD infection with the triplex method, 2 were positives by the Flashdiag®FD method.

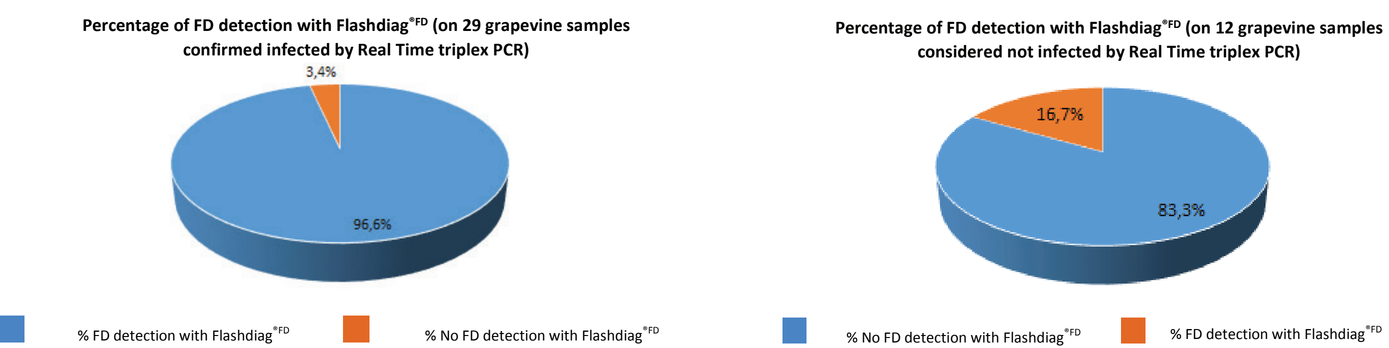

Figure 1: comparison between triplex real time PCR and Flashdiag®FD on the same total nucleic acid extract

The absence of cross detection hypothesis of Flashdiag® FD kits with other phytoplasmas was also tested. This test shows that Flashdiag® FD can also detect map-FD1 (FD70 and FDCAM 05) and map-FD2 (FD92 and FDPEY 05) strains (See figure 2). As it is the case for the triplex real-time PCR test (Pelletier *et al*, 2009), Flashdiag® FD test can also detect two other phytoplasmas of the 16SrV taxonomic group, which are genetically close to the Flavescence Dorée phytoplasma as Palatinate grapevine yellow phytoplasma and '*Candidatus* Phytoplasma rubi'. No other phytoplasma especially '*Candidatus* P. solani', responsible for Bois noir disease, were detected by the Flashdiag® FD.

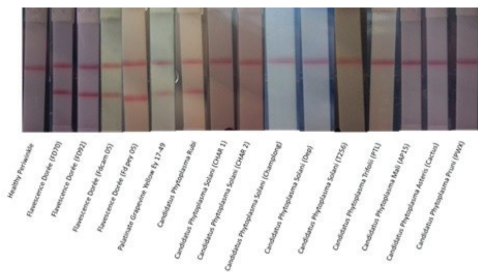

Figure 2: Results of Flashdiag® FD tests for phytoplasma detection on infected periwinkle

CONCLUSION

Preliminary results show that the Flashdiag® FD test sensitivity for FD detection is similar to the official method RT Triplex PCR**.** Furthermore, Flashdiag® FD test can detect different FDP strains as well as the closely related Palatinate Grapevine Yellows (PGY) phytoplasmas, without detecting '*Ca.* P. solani' responsible for Bois Noir disease. A large-scale validation of Flashdiag® FD is on-going in 2015 on 3,000 grapevine samples collected in six French Vineyards.

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PP 44 - Comparison of two PCR technics used in GRBaV diagnostic

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INTRODUCTION

In 2008, a new emerging disease consisting of patches of red blotches along leaf margin, and red veins under the leaf surface were observed in red grape varieties in a few vineyards in Napa Valley, CA. Problem of maturation in red varieties are observed in the infected plants (Calvi 2011). This disease is called Red Blotch and is associated with a virus of the *Geminiviridae* family: Grapevine Red Blotch associated virus – GRBaV – (Thompson 2012). Based on the number of varieties in which GRBaV has been observed and the fact that the virus is transmitted by grafting (Krenz, 2012) it is likely that spread has primarily occurred through propagation material. As it is a really concerning issue for nursery sector in California (Stamps, 2014), Mercier Novatech laboratory decided to start a control strategy based on disease-tested planting stocks. Absence of signs and symptoms in the nursery material require to resort to molecular detection.

Two different all inclusive molecular kits from Qualiplante were used in this study: a conventional PCR kit (ref. PCR. RB-25/100Liq, based on Al Rwahnih et al., 2013) was first used until a qPCR (SYBR-green) kit was developed (ref. SYBR. RB-25/100Liq, internal method based on Krenz et al., 2014).

MATERIALS AND METHODS

Sample preparation: The aim of this study was to compare the GRBaV status of samples from California analyzed with PCR and qPCR. Total DNA of cane woods was extracted using Extraction PLUS kit, (IpadLab, Italy) according to the producer recommendations.

The amount of total DNA in all samples was measured by spectrophotometry. The analysis shows that the amount of DNA was homogenous in all samples (220 ng/µL average).

Conventional PCR: The PCR amplification was done in a 25 µL final volume with 2 µL of extracted DNA. After electrophoresis, EtBr Staining and UV illumination, a sample is positive when a fragment of 557 bp is amplified.

qPCR: The amplification was realized in 20 µL final volume with 3 µL of extracted DNA. 145 cane woods were analyzed by qPCR and 62 DNA samples were chosen to be analyzed by conventional PCR. The value of d(RFU)/dT is directly proportional to the quantity of target DNA in the sample.

RESULTS AND DISCUSSION

Once the results of the qPCR obtained on 145 samples, a conventional PCR was performed for comparison on 62 DNA samples. The analysis shows that under 100 d(RFU)/dT, which correspond to the lowest amount of target DNA, there is no amplification by conventional PCR. Between 100 and 170 d(RFU)/dT, a positive sample in qPCR presents no fragment amplification in conventional PCR. Between 170 and 500 d(RFU)/dT, which correspond to the higher concentration of target DNA, the fragment is randomly amplified in conventional PCR **(Figure 1& 2)**.

Figure 1: Conventional PCR Electrophoresis gel results

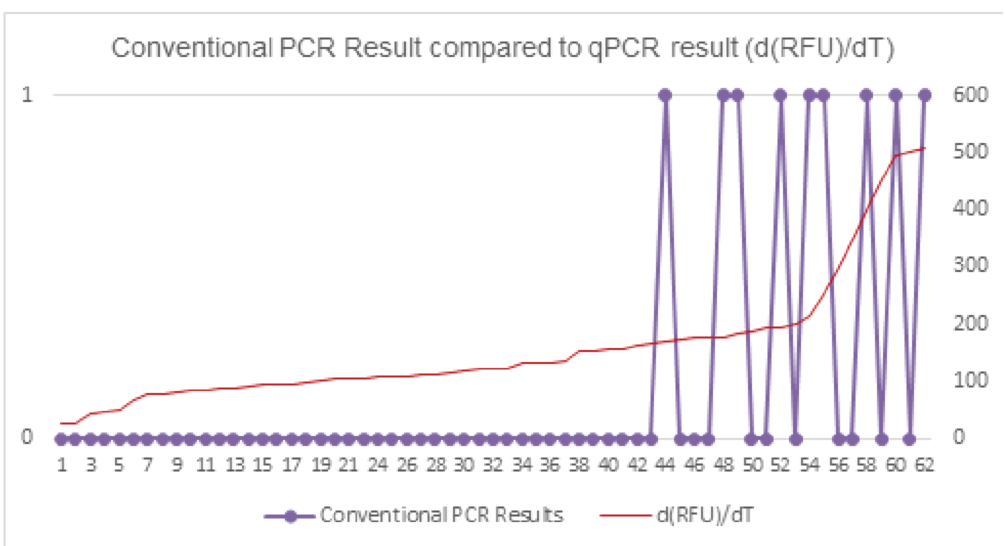

Figure 2: Chart comparing qPCR and conventional PCR results

These results show that using the same extraction technology and without sample dilution, the qPCR is a more accurate technology. It allows detecting positive samples that would have been false negative if analyzed by conventional PCR. qPCR detection is more efficient and can detect slightly infected plants and thus intervene earlier in detection. qPCR method therefore is a better way for a nursery to improve prevention and propagation.

Furthermore, quantification can help evaluating the importance of the plant infection, the distribution and extent of the infection in a plot and potentially how the virus is propagated in the fields.

Mercier Novatech recommends this qPCR technology to control nursery material.

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PP 45 - Effects of *Grapevine red blotch-associated virus* on vine physiology and fruit composition of field grown grapevine cv. Gamay

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INTRODUCTION

Grapevine red blotch-associated virus (GRBaV) is a recently discovered viral pathogen (Krenz et al., 2012). This circular DNA virus has been shown to be associated with red blotch disease (Al Rwahnih et al., 2013). The main symptom of the disease on red cultivars is reddening of leaf blade. This reddening can be confused with symptoms of grapevine leafroll disease. Poojari et al. (2013) have shown that GRBaV infection can be detrimental to the quality of harvest. In this study, we evaluated the impact of GRBaV infection on grapevine physiology and fruit composition of field grown cv. Gamay under cool-climate conditions of Switzerland.

MATERIALS AND METHODS

Two accessions in our grapevine virus collection, Zinfandel (A2V13) and Emperor (A2V18), were found to be infected with GRBaV by PCR using primers developed by Krenz et al. (2014). These two accessions were introduced into our collection in 1985 from UC Davis (California). The Zinfandel accession was tested for the presence of others viruses and appeared only infected by GRBaV. GRBaV from Zinfandel accession was graft-inoculated onto the leafroll indicator *Vitis vinifera* cv. Gamay Rouge de la Loire rooted on 3309 Couderc. Two block of three plants were planted at the Agroscope research station in Nyon in 2000. Fruit composition at harvest and physiological parameters were determined for the 2014 season. Leaf chlorophyll concentrations were estimated using an N-tester chlorophyll meter (Yara, France). Net photosynthesis (P_n), stomatal conductance (g_s) and transpiration (E) were determined on two adult leaves per plant at two time points using the LICOR 6400 XT portable photosynthesis system (Nebraska, USA). Fruit parameters at harvest were measured by NIR spectroscopy (WineScan™,FOSS, US) at Agroscope oenological laboratory.

RESULTS AND DISCUSSIONS

Under cool-climate condition of Switzerland, first symptoms on Gamay leaves appeared during late summer (September). We selected therefore two periods for studying photosynthesis: 18 July (asymptomatic stage) and 8 September (symptomatic stage). The photosynthesis parameters were already impacted in mid-July before development of symptoms. Photosynthesis and transpiration were reduced by around 30% in GRBaV infected vines compared to controls (Table 1). Measurement of photosynthesis during symptomatic stage showed a similar effect.

Table 1. Effects of GRBaV infection on photosynthetic parameters measured on two time points: net photosynthesis (**P_n**, **µmol CO_2** $m^{-2} s^{-1}$), stomatal conductance (**g_s**,(mol CO_2 $m^{-2} s^{-1}$) and transpiration (**E**, mmol H_2O $m^{-2} s^{-1}$). Means with *, **, *** are significantly different at P ≤ 0.05, respectively P ≤ 0.01 and P ≤ 0.001.

	18.7.2014			8.9.2014		
	P_n	g_s	E	P_n	g_s	E
Healthy control	16.7***	0.25***	5.0*	14.8***	0.23*	4.3*
GRBaV infected	12.1***	0.14***	3.30*	11.1***	0.15*	3.3*

Leaf chlorophyll content was monitored repeatedly during the growing season (Fig. 1). GRBaV-infected vines showed consistently reduced leaf chlorophyll content.

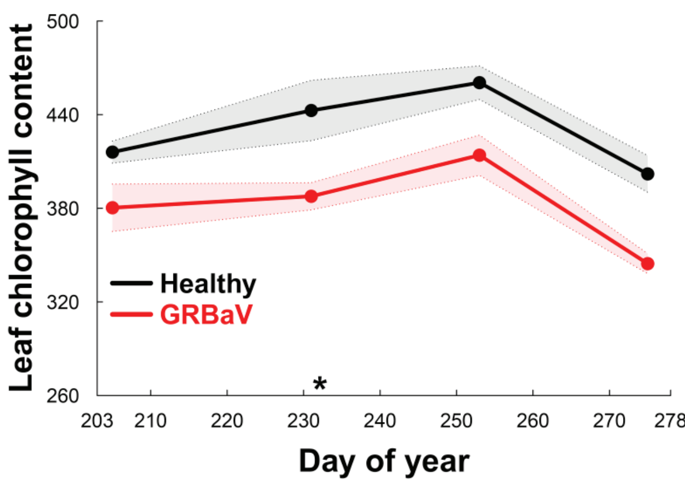

Figure 1. Leaf chlorophyll content in GRBaV-infected and healthy vines at different period during the 2014 season. Each point represents a mean with its 95% confidence intervals. * represents time of veraison.

Results of fruit composition at harvest are displayed in Table 2. The infection by GRBaV was associated with modifications of fruit composition. Fruits from grapevines infected by GRBaV showed lower sugar content and higher pH. The berry acid composition was modified by GRBaV infection: lower tartaric acid content but higher malic acid content in berries.

Table 2. Effects of GRBaV infection on Gamay. Fruit composition at harvest, 2014. Means with **, *** are significantly different at P ≤ 0.01, respectively P ≤ 0.001.

	Soluble solids contents (% Brix)	pH	Titratable acidity (g tartrate/L)	Tartaric acid (g/L)	Malic acid (g/L)
Healthy control	20.5***	3.0***	12.9	8.3***	6.7**
GRBaV infected	18.1***	3.2***	12.8	7.1***	8.2**

In conclusion, the preliminary results presented here showed a clear negative effect on GRBaV infection on vine physiology and fruit composition at harvest of cv. Gamay under cool-climate conditions of Switzerland. Therefore, our results confirm studies and observations made previously about the negative effect of GRBaV on grape production. Considering those observations and the harmful effect of GRBaV on grapevine, virus monitoring in vineyards should be undertaken in order to verify if GRBaV is restricted only to North America and testing for this pathogen should be included in certification program.

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PP 46 - Comparative pathogenic effects of distinct Grapevine fanleaf virus strains on *Vitis vinifera* cvs Gewurztraminer and Chardonnay

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INTRODUCTION

Grapevine fanleaf virus (GFLV) is the main etiological agent of fanleaf disease, one the most severe and widespread virus diseases of grapevine, worldwide. This virus causes serious economic losses (up to 80% yield reduction) and reduces the productive lifespan of vineyards (Andret-Link *et al.*, 2004). Symptoms consist of a progressive degeneration with variable symptoms affecting leaves (yellow mosaic, deformation, vein clearing), canes (short internodes) and clusters (flower abortion, ripeness disturbance). Disease symptom expression depends on the virus isolate, the susceptibility of the variety/rootstock combination, and environmental factors.

GFLV has a bipartite RNA genome (RNA1 and RNA2) and is transmitted by the ectoparasitic nematode, *Xiphinema index*. In recent years, the structure and genetic variability of GFLV populations have been elucidated in several grape-growing regions (Liebenberg *et al.*, 2009, Oliver *et al.*, 2010, Palomares-Rius *et al.*, 2012, Vigne *et al.*, 2009). Mixed infections with genetically distant variants and recombinants are frequent in vineyards, preventing a precise association between genetic variability and symptom expression (Elbeaino *et al.*, 2014).

In order to determine the effect of distinct GFLV isolates on symptoms expression, healthy and infected grapevines were tested in an experimental vineyard. Our main objectives consisted in: i) Monitoring symptom development of five GFLV strains for which the full-length genome sequence was determined, and ii) Analyzing the effect of these strains on fruit yield, as well as on fruit and wine quality. Our findings will shed light on putative viral domains associated to the fanleaf symptoms expression, enabling the selection of GFLV strains with reduced pathogenicity that could benefit future cross-protection experiments aiming at reducing the impact of fanleaf disease in vineyards.

MATERIALS AND METHODS

GFLV strains F13, GHu, B844, CO1(A17b) and CO2(A17d) were isolated from *Vitis vinifera* cvs Muscat de Frontignan, *Gloria Hungariae*, Cabernet franc and Chardonnay respectively (Komar *et al.*, 2008, Legin *et al.*, 1993, Vigne *et al.*, 2005), biologically cloned by multiple passages on herbaceous hosts. They were subsequently transferred to the rootstock Kober 5BB by *in vitro* heterologous grafting.

The experimental vineyard was established in a *X. index*-free plot in 2006 at INRA in Colmar, France. Test plants consisted of *Vitis vinifera* cvs Gewurztraminer (Gw) and Chardonnay (Ch) grafted onto healthy or mono-infected Kober 5BB. For each of the six treatments (infection with one of the five virus strains and mock inoculation), eight Gw vines and eight Ch vines were obtained, for a total of 96 vines that were planted 1 m apart in groups of four vines within three rows.

The nucleotide sequence of the complete genome of the GFLV strains was obtained by RNA Seq (Next Generation Sequencing facility, IGBMC, Illkirch, France) and *de novo* assembly. The verification of the GFLV content in the single-infected vines was confirmed by IC-RT-PCR-RFLP (immunocapture - reverse transcription - polymerase chain reaction - restriction fragment length polymorphism).

Symptoms on plant development, leaves, canes, and clusters were monitored on individual vines. The number of clusters was counted for each plant at harvest, and the clusters weighted. For each treatment, fruit juice chemistry was analyzed and fruits were processed for micro-vinification. Aromatic molecules were detected and quantified by gas chromatography followed by mass spectrometry on the INRA Colmar metabolomics platform. Data were collected from 2012 to 2014. Statistical significance of the results was assessed using the 3.2 R software for ANOVA analyses.

RESULTS AND DISCUSSION

The five GFLV strains displayed at least 9 % nucleotide sequence diversity, regardless of whether RNA1 or RNA2 sequences were analyzed. Unlike strains F13, GHu, CO1(A17b) and CO2(A17d), the genome of B844 is composed of one RNA1 molecule and two genetically distant RNA2 molecules. This original genomic composition with two or more molecular species of RNA1 or RNA2 is novel for GFLV but was already described for other *Secoviridae*, such as *Bean pod mottle virus* strains and *Arabis mosaic virus* (Gu and Ghabrial, 2005, Marmonier *et al.*, 2009).

Regarding symptoms expression, GFLV-B844 causes a severe stunting on Gw cultivar, while the others strains only caused faint mosaic symptoms on Gw leaves. In contrast, all five GFLV strains caused only rare/mild mosaic symptoms on Ch leaves. Significant flower abortion was observed for all strains regardless of the year and cultivar.

Yield impact was similar as already described for other GFLV strains (Walter and Martelli, 1996): crop losses were higher on Ch (- 63%) than on Gw (- 45%), independently of the strain used with the exception of B844 on Gw (- 77%). The effect of the five GFLV strains on wine quality, as measured by must composition, aromatic molecules composition, sensory analyses and comparative tastings, is under way.

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PP 47 - Batch sampling method for the detection of the pathogenic *Agrobacterium vitis*, responsible for crown gall in vines

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INTRODUCTION

The crown gall disease is a serious problem for nursery and vineyards causing growth and yield decline. *Agrobacterium vitis* (*A. vitis*) is the predominant specie inducing tumors on grapevines that can be pathogenic by harboring a Ti plasmid or no-pathogenic (Burr and Otten, 1999). *A. vitis* survives systematically in vines and infected vines may remain asymptomatic until the vine gets injured (Lehoczky, 1968). Thus, an effective diagnosis method for pathogenic strains is needed to control the sanitary status of vines. The objective of this study was to set up a sampling protocol for routine detection on asymptomatic plants. Experiments were conducted onto different vine organs and sampling rates to define a reliable protocol.

MATERIALS AND METHODS

A. vitis detection in roots and woody canes*:* 75 asymptomatic vines from 4 varieties were sampled. All the roots and one woody cane were collected per plant and analyzed separately.

A. vitis distribution in roots*:* 38 symptomatic vines were used to study bacteria distribution in roots. For each plant, all the roots were analyzed individually.

Sampling rates*:* For 6 batches, 2 sampling rates by batch were compared with 1% and 5% of the vines sampled. The number of symptomatic plants was systematically marked but only the asymptomatic plants were used for this analysis. For each plant, all the roots were removed and analyzed globally. The second modality has not been tested for the 2 batches showing the highest levels of symptoms.

Grouping test*:* Batches 1 to 4 were used for this test. For each batch, one root was randomly taken off from 5% of vines. Then, 10 roots were grouped together for analysis. The test was carried out twice.

Bio-PCR: Bacteria were extracted from tissue by crushing 1 g of tissue in 6 mL of sterile water and after 30 minutes, 50 µL of the suspension were plated on AB-Ta medium (Portier P., pers comm). Plates have been incubated at 28°C for 5 days and washed with sterile water. Samples were lysed in boiled water for 10 minutes, cooled on ice and centrifuged. PCR was performed using two primers sets. PehA3/PehA4 amplifies a specific fragment of *A. vitis* (Desquiret V., pers comm) and VCR3/VCF3 amplifies a specific fragment of the Ti plasmid (Kawaguchi et al., 2005).

Samples were all collected in winter and analyzed by Bio-PCR. They were considered infected after analysis when pathogenic *A. vitis* was detected.

RESULTS AND DISCUSSION

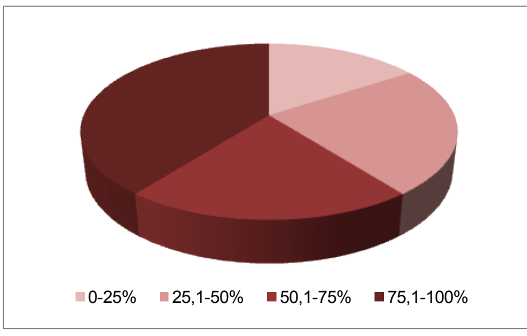

Figure 1: *A. vitis* distribution in roots of 38 symptomatic vines.

Classes of infected roots are expressed in percentages.

A. vitis detection in roots and woody canes of asymptomatic vines

Pathogenic bacteria were detected in 2 plants both onto roots and woody canes. In the other 34 plants, the pathogen was only detected on roots. Roots appeared to be the best organ to detect *A. vitis*. Consequently, we focused our research on roots.

A. vitis distribution in root

The distribution seemed heterogeneous even if a majority of the roots appeared infected when taken from symptomatic plants. Almost 2/3 of the tested vines showed more than 50% (classes 50%-75% and 75%-100%) of the roots infected by the bacteria (Figure 1). Thus, using all the roots for analysis does not appear necessary.

Table 1: Pathogenic *A. vitis* detection in batches depending on the sampling rates. Modality 1: sampling rate = 1%; Modality 2: sampling rate = 5%.

Batches	Symptom of crown gall	% infected samples	
		Modality 1	Modality 2
1	0%	0%	5%
2	0,13%	25%	28%
3	0,93%	0%	40%
4	1,13%	19%	39%
5	3,86%	59%	NT[a]
6	5,86%	86%	NT

[a]üüüüüüü

Comparison of the sampling rates

On table 1, we can notice that the pathogenic bacteria was not detected in 2 batches with a sampling rate of 1% while all the batches were contaminated with a higher sampling rate. So, a sampling rate of 5% seems more adapted for an effective detection.

Table 2: Percentages of infected samples found in 4 batches by the two sampling methods.

Batches	Individual test	Grouping test	
		Repetition 1	Repetition 2
1	5%[a]	12,5%	50,0%
2	28%	100,0%	75,0%
3	40%	87,5%	100,0%
4	39%	100,0%	100,0%

[a] Percentage of infected samples

Grouping test

The test by grouping 10 roots showed a contamination for the 4 batches and the 2 repetitions (Table 2). When a lot was considered as infected by individual test (plant by plant), the lot was as well considered infected by grouping test (10 plants grouped). This technique allows a good level of detection and a reduction of samples number, even for an asymptomatic batch (batch 1).

In this study, a sampling protocol was developed for the detection of infection in asymptomatic vines. For a reliable diagnosis test, samples could be collected by taking one root per plant on 5% of the plants and grouping 10 roots for Bio-PCR analysis. The use of healthy plant is needed to prevent the spread of the pathogen but, as *A. vitis* can survive in dead grape debris in soil, the site selection is also important. *A. vitis* has not been detected in non-vineyard soil (Burr and Otten, 1999). So, planting healthy vines in non-vineyard soil may be an effective control to avoid the contamination of the plants.

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PP 48 - Spatio-temporal epidemiological pattern of Bois noir in a cv. Chardonnay vineyard

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INTRODUCTION

In Europe, one of the most recurrent grapevine yellows is Bois noir (BN), the spread of which is usually endemic (Maixniner, 2011). Over recent decades, frequent bois noir outbreaks have been recorded (Cvckrovic et al., 2014; Murolo et al., 2014; Kosovac et al., 2015), with perturbations to leaf gas exchange, chlorophyll a fluorescence, pigment content and maximum quantum efficiency of photosystem II, with the direct influence of decreased total berry production (Endeshaw et al., 2012).The causal agent of BN is a phytoplasma that belongs to the stolbur group (16SrXII-A subgroup) and recently assigned to '*Candidatus* Phytoplasma solani' (Quaglino *et al*., 2013). It is transmitted mainly by the polyphagous cixiid planthopper *Hyalesthes obsoletus* to a wide range of wild plants such as *Convolvulus arvensis*, *Calystegia sepium*, *Urtica dioica* and several other herbaceous hosts (Langer and Maixner, 2004). These all thus represent potential inoculum sources. On the other hand, grapevine is only occasionally infected by *H. obsoletus* and can be considered a dead-end host for the stolbur phytoplasma.

Several recent studies focused on spatial BN analysis, improving the knowledge on its epidemiology (Marchi et al., 2011; Murolo et al., 2014) and investigating the possible role of host plants and insect vectors in the spread of phytoplasma diseases (Navratil et al., 2009; Mori et al., 2014). The aim of this study were therefore to describe the epidemiologica pattern of Bois noir, recording the incidence and severity inside the vineyard during two years of surveys.

MATERIALS AND METHODS

The trials were carried out through 2011 and 2012 in a cv. Chardonnay commercial vineyard in Marche region (Italy). Visual inspections were carried out at middle of September in 2011 and 2012 to assess the incidence of BN. In order to estimate the disease severity, for the symptomatic plants we used an empirical scale (1-5), where 1 = plant; showing 1–2 leaves with symptoms; 2 = plant showing more than 2 leaves with symptoms on one shoot; 3 = plant with leaves with symptoms on more than one shoot; and 4 = plant with more than 50% of canopy with symptoms.

We elaborated bidimensional maps for 2011 and 2012 by SYSTAT programme, plotting symptoms and symptomless/ healthy vines in order to spatially monitor the epidemiology of bois noir. The occurrence of disease gradients within the vineyard for these BN-affected grapevines was studied. For this purpose, the percentages of diseased plants in the 13 rows and the 24 plots across each row were calculated. The percentages of diseased plants in each plot were plotted and the regression curve calculated in the vineyard for 2011 and 2012. Using PASSAGE software, v. 2 (Rosenberg and Anderson, 2011), the aggregation of adjacent vines and the more complex spatial relationships over longer distances were examined by Morisita's index.

The data of symptom severity (z) that were recorded in 2011 and 2012 were defined with respect to plant position (x, y). The SYSTAT software first computes its own square grid of interpolated or directly estimated values. From this grid, contours were followed using the method of Lodwick and Whittle (1970), combined with linear interpolation. The plot automatically determines the number of contours to draw, so that the surface is delineated and the contour labels can be characterized by different colors.

RESULTS AND DISCUSSION

During the surveys carried out in September, we recorded 775 symptomatic vines 2011 and 400 vines in 2012. In September in Central-Eastern Italy, symptoms appear unambiguously, and the titre of phytoplasma is generally high, which allows the easy and reliable detection of plants with symptoms (Murolo *et al*., 2014). Using the SYSTAT software, the positions of the healthy vines and vines with symptoms recorded in 2011 and 2012 were plotted, to obtain a bidimensional map for each year of investigation. In both years, there was a higher frequency of vines with symptoms along the borders of the vineyard than in its central part. The regression curve that overlapped the percentage of diseased vines compared with the distance from the border of the rows was in the form of a binomial curve in both 2011 and in 2012. A similar situation

was observed in vineyards for the epidemiology of Flavescence dorée (Pavan *et al*., 2012). The occurrence of decreasing gradients of BN-infected grapevines from the vineyard borders shows that external sources of infectious *H. obsoletus*, or other potential vectors, have an important role in the epidemiology of BN (Maixner, 2011; Mori et al., 2012). From the analysis of the dispersion index, the distribution of the vines with symptoms in both 2011 and 2012 showed a uniform pattern. In particular, Morisita's index were <1.The indices of dispersion show that the distribution of the vines with symptoms follows a uniform or regular pattern, without clustering of infected plants or clustering of healthy plants. This applies across the data recorded for both of these years, and even in the season when the BN incidence was particularly high. Indeed, it is well known that the spread of BN in the vineyard does not occur from plant to plant, but is instead mediated by the weeds that represent potential inoculum sources. Considering the distribution of vines in the vineyard according to the severity of the BN leaf symptoms, the construction of the two-dimensional contour maps provides a clearer graphical visualization of the vines that were more severely affected by BN along the borders of the vineyards in 2011, when the incidence of vines with symptoms was higher. This picture of disease severity appears to confirm a natural source of inoculum and the activity of potential vectors in spreading BN in this vineyard.

These data can also contribute to better management of phytoplasma disease, together with weed control and increased plant resistance, thus also further promoting sustainable agricultural practices (Romanazzi et al., 2013).

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PP 49 - Virus infections and sanitation of ancient native grapevine cultivars from Apulia

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INTRODUCTION

In the frame of the EU rural development program 2007/2013, the Apulian Regional government financed the project Re.Ge.Vi.P. ("Recovery of Apulian grape germplasm"), aiming at the rescue of ancient native grapevine cultivars and biotypes. Thus, in 2013/2014, 152 grape selections, comprising biotypes and more than 75 putative cultivars, the majority of which described in historical pre-philloxera reports, were recovered throughout the Apulian territory. Given the lack of information on the sanitary status of these neglected grapevine types, investigations were initiated for the occurrence of viruses regulated in the Italian grapevine certification system and their elimination attempted.

MATERIALS AND METHODS

Dormant canes of 80 native table and wine grapes were used for virus screening. Cortical scrapings from these accessions were tested by ELISA for the presence of *Grapevine virus A* (GVA), *Grapevine virus B* (GVB), *Grapevine leafroll-associated virus 1*, *2*, *3* (GLRaV-1, -2, -3), *Grapevine fanleaf virus* (GFLV), *Grapevine fleck virus* (GFkV) and *Arabis mosaic virus* (ArMV), using commercial kits (Agritest, Italy).

Selected accessions were propagated and those positive for single or multiple virus infections were submitted to sanitation. According to the virus species and based on previous experiences (Bottalico *et al.,* 2003), meristem tip culture and/or termotherapy were carried out on *in vitro*-grown explants.

Overall, 598 meristem-derived grape plantlets were transferred to the greenhouse after acclimatization and 119 of them were tested by RT-PCR for the same virus panel looked for in mother plants. Time for testing was determined according to the growth rate of each grape accession. It always exceeded 60 days from transferring to the greenhouse. Whenever possible, at least three plantlets deriving from distinct apex excisions, were tested for each accession. Total RNA extraction and cDNA synthesis were done according to Faggioli *et al.* (2012). For PCR amplification, primers designed by Gambino and Gribaudo (2006) were used in a single, instead of multiplex reaction.

RESULTS AND DISCUSSION

Of 80 samples tested by ELISA, each corresponding to a distinct putative cultivar, 68 (85%) were infected by viruses included in Italian (DM 07/07/2006 and DM 24/06/2008) regulations (Tab. 1). Mixed infections were found in 65% of the vines, some of which hosted up to five viruses, (i.e. table grapes "Beccarosa" and "Corniola Bianca", from Bari's countryside, and wine cultivars "Malvagia Nera" and "Tinturino", from the Gargano promontory). GLRaV-3 was the most frequently detected virus (71.3%), followed by GVA (42.5%). The prevalence of GLRaV-3 was consistent with previous findings (Cabaleiro *et al.*, 2006), and its close association with GVA was in line with the observation by Zorloni *et al.* (2006), since these viruses share the same pseudococcid mealybug vectors. Other leafroll-associated virus diseases were found at a lower rate of occurrence, i.e. 22.5% (GLRaV-1) and 5.0% (GLRaV-2). The frequency of infectious degeneration agents was of 28.8% for GFLV, whereas all samples tested were ArMV-negative. This virus is known to be rare in the Mediterranean area, also because the low occurrence of its vector, *Xyphinema diversicaudatum* (EFSA, 2013). GFkV had a high incidence (45%). The present legislation requires its absence only in certified rootstocks. GVB was never detected by ELISA in the material analysed pre-sanitation, but it popped up in a sanitized plantlet. Whether this indicates a lower sensitivity of ELISA with respect to PCR or a mistake in assessing the ELISA result remains to be established. Meristem tip culture and

thermotherapy successfully eliminated viruses in 79% of the 119 RT-PCR tested plantlets (Tab. 1). Of the 25 accessions still infected after treatment, most were positive to GFLV and GFkV (8 each), 6 to GVA, one each to GVB, GLRaV-2 and GLRaV-3. The necessity for producing multiple explants as a source of healthy propagation material (Sim *et al.*, 2012), was confirmed by the contemporary detection in four cultivars of infected and healthy plants derived from explants excised from the same source. The persistence of GFLV, GFkV, GVA, GVB infections respectively in 8, 2, 2 and 1 treated plantlets, which was not detected by the preliminary ELISA screening of source plants, underlines the higher sensitivity of RT-PCR detecting these viruses, although ELISA remains a valid protocol for large-scale surveys (Faggioli *et al.*, 2012).

Table 1. Virus infection frequency as ascertained by ELISA test on source plants, and RT-PCR test on propagated plantlets, respectively prior or after sanitation treatment.

ELISA (pre-sanitation)			RT-PCR (post-sanitation)		
VIRUS	n	%	VIRUS	n	%
GVA	34	42.5	GVA	6	5.0
GVB	0	0.0	GVB	1	0.8
GLRaV-1	18	22.5	GLRaV-1	0	0.0
GLRaV-2	4	5.0	GLRaV-2	1	0.8
GLRaV-3	57	71.3	GLRaV-3	1	0.8
GFLV	23	28.8	GFLV	8	6.7
ArMV	0	0.0	ArMV	0	0.0
GFkV	36	45.0	GFkV	8	6.7
Infected	68	85.0	Infected	25	21.0
Single Infection	16	20.0	Single Infection	25	21.0
Multiple Infection	52	65.0	Multiple Infection	0	0.0
Healthy	12	15.0	Healthy	94	79.0
Tested Mother Plants	**80**	**-**	**Tested Plantlets**	**119**	**-**

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PP 50 - Sanitation of a Tunisian grapevine 'Hencha' cv. *via* direct somatic embryogenesis

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INTRODUCTION

Tunisia possesses a rich patrimony which gathers around 35 distinct cultivars; each local cultivar was at least infected by two to three viruses (Mahfoudhi et al., 2014). Viral diseases are reported to cause several detrimental effects on grape production. Viruses are disseminated mainly through vegetative propagation and grafting. Therefore, the use of virus-free vines for multiplication and vineyard planting is highly desirable. Actually, sanitation programs of grapevines are being complemented with the development of *in vitro* regeneration systems. In fact, somatic embryogenesis was effective in eliminating fanleaf and leafroll-associated viruses from grapevines (Goussard et al., 1991). This technique was also used for the elimination of grapevine stem pitting associated virus (Gribaudo et al., 2006). This technique was applied to a Tunisian grapevine 'Hencha' cultivar in an attempt to eliminate GVA and GRSPaV viruses.

MATERIALS AND METHODS

Proliferation of embryogenic cultures: Inflorescences at stage H of Bagiollini were obtained by forcing technique from fruity-cutting (Fig. 1). Somatic embryos were directly induced from filaments of 'Hencha' cultivar according to (Bouamama et al., 2007). Primary somatic embryos, proliferation of secondary embryogenesis as well as maturation stage were conducted on (Chée and Pool,1987) based medium supplemented with 2 $mg.l^{-1}$ 2,4-D and 2.5 $mg.l^{-1}$ TDZ, under darkness. Germination of mature somatic embryos was realized on MS with 0.1% charcoal. Somaplants acclimatized since one year were subjected to serological, molecular and cytometric analysis.

Molecular analysis of somaplants: The sanitary status of 'Hencha' cultivar was assayed by ELISA and RT-PCR. Samples from Hencha cultivar were collected from the Center of Biotechnology of Borj-Cédria Extract obtained from mature canes were tested by sandwich enzyme-linked immunosorbent assay (ELISA) for the presence of GVA, GLRaV-1, -2, -3, GFLV, GFkV ArMV as indicated by (Mahfoudhi et al., 2014). Total RNA was tested for the presence of GVA, GRSPaV, GFLV, ArMV, GLRaV1, 2, 3, GFkV and GVB using specific primers as indicated by (Mahfoudhi et al., 2014),

RESULTS AND DISCUSSIONS

This work deal with, the elimination of grapevine viruses, *via* direct somatic embryogenesis and the propagation of healthy material, which is the main purpose of certification program.

Hencha is a white cultivar originated from the south of Tunisia, which present an interesting organoleptic characteristic of their grapes. GVA was detected by double antibody sandwich-indirect-enzyme-linked immunosorbent assay (DASI-ELISA) in woody mother plants of Hencha cultivar. Transcriptase reverse-Polymerase chain reaction analysis proved that Hencha cultivar is infected by GVA and GRSPa-V.

On the other hand, about 13% of filaments produced direct somatic embryos after two weeks of cultivation (Fig. 2), when cultivated on 2,4-D and TDZ. Primary somatic embryos induced directly were visualized on scanning electron microscopy (Fig. 3). Somatic embryos were reoriented towards an indirect somatic embryogenesis on the same medium under darkness (Fig. 4). Embryogenic calli developed into mature somatic embryos after nine months of cultivation (Fig. 5). Acclimatized somaplants grew in the greenhouse and were maintained as virus-free plants (Fig. 6).

Results of transcriptase reverse-PCR, using virus-specific oligonucleotide primers on 50 somaplants, showed that 100% of somaplants developed *in vitro* were free from grapevine virus A and grapevine stem pitting associated virus. (Gribaudo et al., 2006) reported that the presence of GRSPa-V alone induces few rugose wood symptoms on grapevine, while, the presence of other viruses such as GVA may be required for rugose wood symptoms to occur. The double infection can

cause severe damages to infected grapevines plants. So, that is why somatic embryogenesis seems to be a very promising technique for the production of healthy grapevine stocks.

Actually, we are developing a cytometric analysis on 50 acclimatized somaplants in order to confirm that they are true to type to their parental plants.

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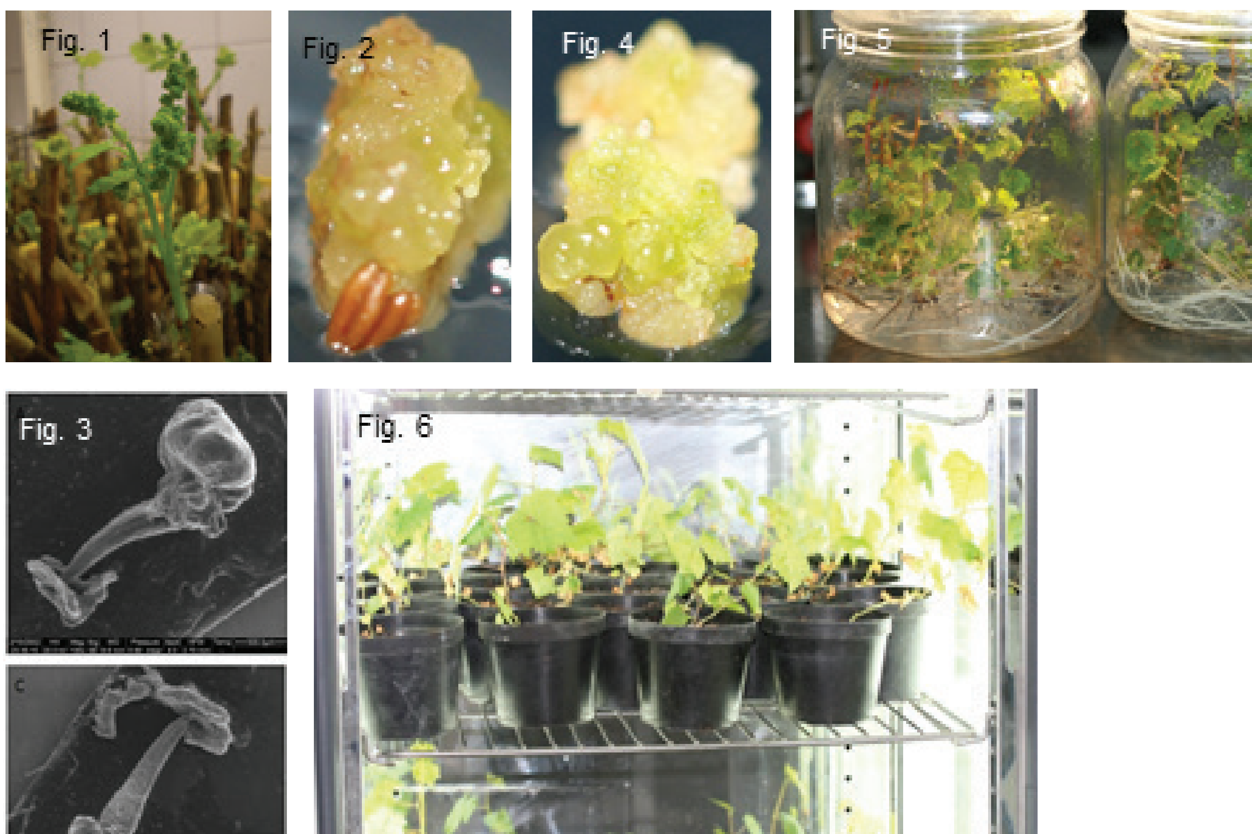

In vitro regeneration of grapevine somaplants for virus elimination.
Figure 1: fruity-cutting. Direct primary somatic embryos.
Figure 3: visualization of somatic embryos by scanning electron microscopy.
Figure 4: secondary somatic embryogenesis.
Figure 5: germination of somatic embryos.
Figure 6: acclimatization of somaplants

PP 51 - Production of *Grapevine Pinot gris virus*-free germplasm: techniques and tools

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INTRODUCTION

Grapevine Pinot gris virus (GPGV) is a recently described trichovirus (Giampetruzzi et al., 2012) seemingly associated with symptoms of leaf mottling and deformation. Independent studies considering virus isolates from two Italian viticultural Regions, reported the existence of GPGV symptomless or symptomatic isolates, which are distinguishable based on their phylogeny (Saldarelli et al., 2015) or virus titer in the infected vines (Bianchi et al., 2015). Since the initial description of GPGV sequence in Italy, the virus has been detected in Slovak and Czech Republics, Slovenia, France, Greece (Martelli, 2014) and Turkey (personal information). Besides Europe GPGV was reported in South Korea (Cho et al., 2013) on the *Vitis labrusca* cv. Tamnara, which showed symptoms similar to berry necrosis. GPGV detection is mainly accomplished by RT-PCR using different sets of primers. Efficient management of virus disease relies on virus exclusion from the vineyards. Preventive strategies to achieve this goal consist mainly on vector control and the production of sanitized plant propagation material. In addition, the availability of reliable and easy-to-use tools for diagnosis are fundamental to support these actions. We started the production of GPGV-free grapevine plant propagation material of the cv. Traminer and Pinot gris by evaluating different sanitation techniques. In parallel, an antiserum against the GPGV coat protein (CP), expressed and purified from *Escherichia coli,* was produced. The present abstract reports the results of these preliminary activities.

MATERIALS AND METHODS

Grapevine sources. GPGV-infected vines belonging to the cvs. Pinot gris and Traminer and showing symptoms of leaf mottling and deformation were selected for sanitation. Additional GPGV-infected vines and grapevine seedlings were included in the trial to evaluate the performance of the anti-GPGV CP serum. The sanitary status of all the vines used throughout the study was assessed by RT-PCR against *Grapevine virus A* (GVA), *Grapevine virus B* (GVB), *Grapevine leafroll-associated virus 1*, *2*, *3* (GLRaV-1, -2, -3), *Grapevine fanleaf virus* (GFLV), *Grapevine fleck virus* (GFkV) and *Arabis mosaic virus* (ArMV). Total RNA extraction and cDNA synthesis were according to the validated protocol described by Faggioli et al. (2012). PCR detection was performed with primers designed by Gambino and Gribaudo (2006), used in single, instead of multiplex reaction.

Production and use of an antiserum against GPGV CP. GPGV CP was expressed and purified from *E. coli* using the pGEX system (GE Healthcare, UK). The glutathione-S-transferase (GST)-tagged CP was used to immunize a rabbit (GeneCust, Luxembourg). Collected antiserum was cleared of the GST-tag specific antibodies and used for serological detection at 1: 2000 dilution. Western blot analysis was accomplished on protein extracts from grapevine leaf tissues, which were ground in Laemmli 1Xbuffer in a 1:5 w/v ratio and separated in 12% SDS-PAGE. Membrane blotting and detection of immune-complexes was performed according to standard techniques.

Plant sanitation: Based on previous experiences (Bottalico et al., 2003), *in vitro* meristem tip culture and/or thermotherapy were carried out on *in vitro*-grown explants or potted plants. Thermotherapy-treated vines were maintained for two to four months at 34°C or 38°C before meristem excision.

RESULTS AND DISCUSSION

The anti-GPGV CP serum failed to detect the virus by ELISA due to a high background reaction. Conversely, in Western blot analysis the serum clearly identified a *ca.* 22 kDa denatured band, likely corresponding to the GPGV CP, only in plants showing specific symptoms of leaf mottling and deformation and infected by GPGV as assessed by RT-PCR (**Table 1**). A more extensive survey is needed to evaluate performances of this serum with respect to different GPGV isolates. Plant sanitation by meristem tip culture was accomplished with or without thermotherapy. The cv. Traminer better performed after sanitation whereas P. gris vines did not survive the process. Six potted-transferred vines, either subjected [Tr5A(2B); Tr5A(16A); TR1A-1R; TR1A-3R] or not [Tr5A(6B); Tr5A(17A)] to thermotherapy, proved to be GPGV-free by RT-PCR and WB assays, after an acclimatization period of 6 [Tr5A(2B); Tr5A(6B); TR1A(1R) and TR1A(3R)] or 3 [Tr5A(16A) and

Tr5A(17A)] months. All sanitized vines did not show symptoms in a range of time of 6 months of observations. The present work, beside describing the development of an anti-GPGVCP serum and producing GPGV-free Traminer vines, further supports the association of GPGV to symptoms of leaf mottling and deformation.

ID.	Cultivar	Clone code	GPGV symptoms	GPGV detection	
				RT-PCR	WB
PG1A	Pinot gris	ZA505-1A	+	+	+
Tr4A	Traminer	FI4A	+	+	+
Tr5A	Traminer	FI5A	+	+	+
Tr1A	Traminer	FI1A	+	+	+
GTr	Gold Traminer	GTr	+	+	+
PG3A	Pinot gris	ZA505-3A	+	+	+
PGUK	Pinot gris	PGUnknown	+	+	+
T152	Teroldego	T152	+	+	+
18K	Muscat	MG18K	-	-	-
DON	Rootstock	DONS35	-	-	-
Tr5A (2B)	Traminer	Tr5A (2B) sanitized	-	-	-
Tr5A (6B)	Traminer	Tr5A (6B) sanitized	-	-	-
Tr5A (16A)	Traminer	Tr5A (16A) sanitized	-	-	-
Tr5A (17A)	Traminer	Tr5A (17A) sanitized	-	-	-
TR1A (1R)	Traminer	TR1A (1R) sanitized	-	-	-
TR1A (3R)	Traminer	TR1A (3R) sanitized	-	-	-
S1	Grape seedling	S1	-	-	-
S2	Grape seedling	S2	-	-	-
S3	Grape seedling	S3	-	-	-
S4	Grape seedling	S4	-	-	-

Table 1. GPGV detection by RT-PCR and Western blot on field grown, sanitized or grapevine seedlings. Positive (+) and negative (-) RT_PCR amplification or CP serological detection by WB are indicated.

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PP 52 - Investigations on distribution patterns of mealy bugs and grapevine leafroll in vineyards near the river Nahe in Southern Germany

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INTRODUCTION

Grapevine leafroll is a long known and economically important disease of *Vitis vinifera.* American and Asian *Vitis* species are susceptible to infection but show no typical symptoms. Red or reddish-purple discolorations in interveinal areas can be observed on most red-berried *V. vinifera* varieties, whereas many white-berried *V. vinifera* cultivars show yellowing or chlorotic mottling of interveinal areas of leaves. Downward rolling of leaf margins is typical for all cultivars at a later stage of the season (Naidu *et al.*, 2014). Several morphologically similar grapevine leafroll-associated viruses (GLRaVs) have been described. They all belong to distinct genera in the family *Closteroviridae*. GLRaV-1, -3, and -4 are members of the genus Ampelovirus, GLRaV-2 falls within the genus Closterovirus, and GLRaV-7 belongs to the genus Velarivirus. Several other GLRaVs (-5, -6, -9, -Pr, -De, and –Car) were recently recognized as genetically divergent strains of GLRaV-4 (Martelli *et al.*, 2012).

Mealybugs and scale insects are able to transmit GLRaVs belonging to the genus Ampelovirus.There are no known insect vectors for GLRaV-2 and -7. Unless mealybugs/scale insects can be dispersed long distances by wind or other means, the natural spread of these viruses in orchards is due to the limited mobility of the insects over short distances. Depending if the source of infection results from within a vineyard or from sources outside, differences in spatiotemporal spreading can be observed (Naidu *et al*., 2014).

MATERIALS AND METHODS

Four vineyards in the German vine growing region of the river Nahe with the varieties Cabernet Mitos, Pinot Gris, Ortega and Pinot Noir were observed for symptoms and tested in Sept. 2013 and Feb. 2014 by multiplex PCR for *Arabis mosaic virus* (ArMV), *Grapevine fanleaf virus* (GFLV), *Grapevine virus A* (GVA), *Grapevine virus B* (GVB), *Rupestris stem pitting-associated virus* (RSPaV), *Grapevine fleck virus* (GFkV), and *Grapevine leafroll-associated virus* -1, -2, and -3 (GLRaV-1, -2, and -3) according to the method described by Gambino & Gribaudo (2006). The primers for GLRaV 1 were modified according to Le Maguet (2012). The vector for GLRaV-1, -3, and -4, and GVA, *Phenacoccus aceris* (Le Maguet *et al.*, 2012), was found frequently in the four orchards. In the two adjacent vineyards at Wallhausen only the first neighboring rows were sampled to check if clusters of virus symptomatic plants exist across the vineyard border, which would be an indicator for virus transmission by a non-aerial vector.

The two vineyards at Mandel were sampled in a raster so that every 16th vine in a row was sampled in the Pinot Noir– and every 12th in the Ortega plot to obtain a picture of the patchiness of symptomatic vines across the whole plot.

RESULTS AND DISCUSSION

Samples from the four vineyards were tested by multiplex PCR for the occurrence of the nine viruses mentioned. In three vineyards the rates of infections were greater than 90%, with the majority of vines mixed infected with GLRaV-1 and GVA (Table 1). GLRaV-3 and other viruses were only found in few incidences. In these vineyards the high rate of infection did not allow for any conclusion on virus transmission by mealybugs even though the vector was present. In the 14 year old Pinot Noir vineyard at Mandel the infection rate was much lower at only 23 % and clearly defined clustering of symptomatic plants together with the vector *P. aceris* was observed (see Fig.1). It is highly probably that the observations in this plot are the result of vector transmission of at least GLRaV-1 by *P. aceris*.

This is the first report under German field conditions of GLRaV-1 infected vines found in clusters. The role of *P. aceris* as a virus vector in German vineyards should be newly evaluated. While scale insects until now where only considered as occasional secondary pests by Hoffmann (2002), we now have to reevaluate the pest status of scale insects in German viticulture.

Table 1. Summary of viral infections in four different vineyards of the winegrowing region Nahe.

variety	Cabernet Mitos	Pinot Gris	Ortega	Pinot Noir
Total # vines	84	82	103	70
# PCR negative for viruses	6	5	1	54
GLRaV-1	16	14	51	15
GLRaV-1 + GVA	61	54	35	1
GLRaV-1 + -3	0	3	1	0
GLRaV-1 + -3, + GVA	1	6	1	0
GLRaV-1 + GFkV	0	0	8	0
GLRaV1 + GVA + GFkV	0	0	1	0
GLRaV1 + GVA + RSPaV	0	0	3	0
GVB	0	0	0	0
GFLV	0	0	0	0
GLRaV 3	0	0	1	0
GLRaV 3 + GVA	0	0	1	0
GFkV	0	0	0	0
RSPaV	0	0	0	0
ArMV	0	0	0	0
Leafroll: % positive samples	**93**	**94**	**99**	**23**

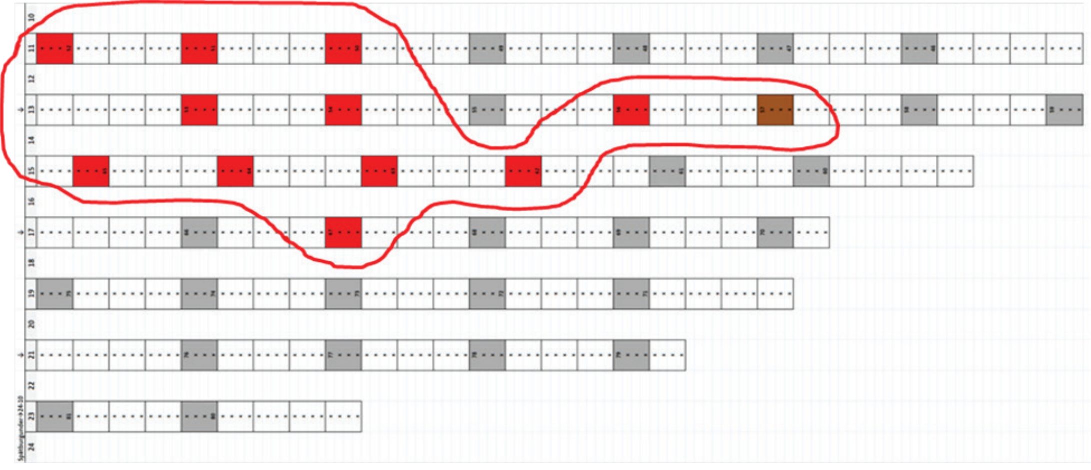

Figure1: Spatial pattern of GLRaV-1 and GVA infection in a Pinot Noir vineyard at Mandel/Nahe (2014).

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